SOYBEAN LUNASIN MEDIATES COLON CARCINOGENESIS BY INDUCING APOPTOSIS AND PREVENTING OUTGROWTH OF METASTASIS

BY

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DISSERTATION

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Colorectal cancer (CRC) is the third most common cancer worldwide. Various factors such as age, lifestyle and dietary patterns affect the risk of having CRC. Epidemiological studies showed a chemopreventive effect of soy consumption against CRC. However, which component(s) of soybean is associated with this reduced risk is not yet fully delineated. The objective of this research was to evaluate the anti-colon cancer potential of lunasin isolated from defatted soybean flour using \textit{in vitro} and \textit{in vivo} models of CRC. Lunasin was isolated from defatted soybean flour by a combination of different chromatographic and ultrafiltration techniques. The anti-colon cancer potential of lunasin was determined using different human colon cancer cell lines \textit{in vitro} and a CRC liver metastasis model \textit{in vivo}. Lunasin caused cytotoxicity to different human colon cancer cells with an IC$_{50}$ value of 13.0, 21.6, 26.3 and 61.7 µM for KM12L4, RKO, HCT-116 and HT-29 human colon cancer cells, respectively. This cytotoxicity correlated with the expression of the $\alpha_5$ integrin on human colon cancer cells with a correlation coefficient of 0.78. The mechanism involved in the cytotoxic effect of lunasin was through cell cycle arrest and induction of the mitochondrial pathway of apoptosis. In KM12L4 human colon cancer cells, lunasin caused a G2/M phase arrest increasing the percentage of cells at G2/M phase from 12% (PBS-treated) to 24% (treated with 10 µM lunasin). This arrest was attributed to the capability of lunasin to increase the expression
of cyclin dependent kinase inhibitors p21 and p27. At 10 µM, lunasin increased the expression of p21 and p27 in KM12L4 colon cancer cells by 2.2- and 2.3-fold, respectively. Flow cytometric analysis showed that lunasin at 10 µM increased the percentage of cells undergoing apoptosis from 13.6% to 24.7%. This is further supported by fluorescence microscopic analysis of KM12L4 cells treated with 10 µM lunasin showing chromatin condensation and DNA fragmentation. The mechanism involved is through modification of proteins involved in the mitochondrial pathway of apoptosis in KM12L4 cells as 10 µM lunasin reduced the expression of the anti-apoptotic Bcl-2 protein by 2-fold and increased the expression of the pro-apoptotic proteins Bax, cytochrome c and nuclear clusterin by 2.2-, 2.1- and 2.3- fold, respectively. This led to increased expression and activity of the executioner of apoptosis, caspase-3 by 1.8- and 2.3-fold, respectively. This pro-apoptotic property of lunasin can be attributed to its capability to internalize into the cytoplasm and nucleus of colon cancer cells 24 h and 72 h after treatment, respectively. In addition, lunasin mediated metastasis of colon cancer cells in vitro by inhibiting the focal adhesion kinase activation thereby reducing expression of extracellular regulated kinase and nuclear factor kappa B and finally inhibiting migration of colon cancer cells. In KM12L4 colon cancer cells, 10 µM lunasin resulted in the reduction of phosphorylation of focal adhesion kinase and extracellular regulated kinase by 2.5-fold, resulting in the reduced nuclear translocation of p50 and p65 NF-κB subunits by 3.8- and 1.4-fold, respectively. In an in vivo model of CRC liver metastasis, daily intraperitoneal administration of lunasin at 4 mg/kg body weight resulted in the inhibition of KM12L4 liver metastasis as shown by the reduction of the number of liver metastases from 28 (PBS-treated) to 14 (lunasin-treated, P = 0.047) and
reduction in tumor burden as measured by liver weight/body weight from 0.13 (PBS-treated) to 0.10 (lunasin-treated, \( P = 0.039 \)). Moreover, lunasin potentiated the anti-metastatic effect of the chemotherapeutic drug oxaliplatin given at 5 mg/kg body weight twice per week. Lunasin and oxaliplatin combination resulted in a more potent inhibition of outgrowth of KM12L4 cell metastases to the liver reducing the number of liver metastases by 6-fold and reducing the tumor burden in the liver by 3-fold when compared to PBS-treated group. This can be attributed by the capability of lunasin and oxaliplatin to reduce expression of proliferating cell nuclear antigen in liver-tumor tissue as measured by immunohistochemical staining. The results of this research for the first time demonstrated the anti-colon cancer potential of lunasin isolated from defatted soybean flour which might contribute to the chemopreventive effect of soybean in CRC as seen in different epidemiological studies. In conclusion, lunasin isolated from defatted soybean flour mediated colon carcinogenesis by inducing apoptosis and preventing outgrowth of metastasis. We suggest that the results of this research serve as a basis for further study on the chemopreventive effect of lunasin against CRC and a possible adjuvant role for lunasin in therapy of patients with metastatic CRC.
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>BBI</td>
<td>Bowman-Birk inhibitor</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin dependent kinase inhibitor</td>
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<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
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<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimum essential medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>H and E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration needed to inhibit 50% of cell growth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IEC</td>
<td>Ion exchange chromatography</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MS-MS</td>
<td>Tandem mass spectrometry</td>
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<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>nCLU</td>
<td>Nuclear clusterin isoform</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of kappa-B</td>
</tr>
<tr>
<td>Ox</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>OxR</td>
<td>Oxaliplatin resistant</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PES</td>
<td>Phenazine ethosulfate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q-FF</td>
<td>Q-Sepharose fast flow</td>
</tr>
<tr>
<td>Q-Tof</td>
<td>Quadruple time of flight</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immunoprecipitation assay</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
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x
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline-Tween 20</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
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CHAPTER 1

OVERVIEW

Cancer is a world-wide problem. It is the leading cause of death in developed
countries and the second leading cause of death in developing countries [1]. Based on the
GLOBOCAN 2008 estimates, approximately 12.7 million cases and 7.6 million cancer-
related deaths occurred in 2008 [2,3]. Lifestyle and dietary factors are considered to be
the major determinants of premature mortality and chronic diseases such as cancer [4].
For instance, obesity, physical inactivity, alcohol consumption and poor diet are major
risk factors for premature death and cancer [5]. On the other hand, the World Cancer
Research Fund concluded that a diet rich in fruit and vegetables showed ‘probable’ or
‘limited-suggestive’ evidence in lowering cancer risk [6]. Epidemiological studies
suggested that consumption of soy and soy products is associated with lowering risks of
cancer including cancer of the breast [7], prostate [8, 9] and colon and rectum [10,11].
This chemopreventive effect of soybean against cancer can be attributed to different
bioactive compounds present in soybean including isoflavones, saponins, lignans, phytic
acid and bioactive peptides and proteins [12-16].

Colorectal cancer (CRC) is the third and the second most commonly diagnosed
cancer in males and females, respectively, with over 1.2 million new cases and 608,700
deaths estimated to have occurred in 2008 worldwide [3]. It entails a multi-stage process
transforming normal tissue into invasive cancer controlled by many genetic and
environmental factors involving activation of oncogenes and inactivation of tumor
suppressor genes. In its multistep process, somatic mutations develop in adenomatous
polyposis coli gene, p53 tumor suppressor gene, K-Ras oncogene and various genes that
mediate DNA mismatch repair [17]. The survival rate of patients with cancer is largely
determined by the stage of diagnosis. In CRC, patients diagnosed at early stage of the
disease with only localized growth of the cancer; a 5-year survival rate is around 95%.
The survival rate is reduced to ~11% for patients diagnosed at stage IV cancer where the
tumor metastasized to distant lymph nodes and organs [18]. More than 90% of cancer-
associated deaths are due to metastasis [19]. Metastasis develops in several steps namely
local invasion, intravasation, survival in the circulation, extravasation and colonization.
During these steps, cancer cells acquire unlimited proliferative potential, oncogenic
transformation, ability to surmount natural barriers to spread, distant organ infiltration
and colonization [20]. The prominent feature of metastasis is its capability to colonize
specific organ sites. In metastatic CRC, the mesenteric circulation and perforated
capillaries of the liver facilitate hepatic metastasis [21, 22]. The transformation of colon
cancer cells into an invasive carcinoma involves mutations in the Wnt pathway, followed
by K-Ras activation, PI3K activation, p53 inactivation and loss of the transforming
growth factor-β tumor suppressor pathway. The remaining mutations required to institute
metastasis as well as the time required for CRC tumors to metastasized is not well
understood and differs among individuals with CRC, suggesting the need for
personalized management for patients [23, 24]. Diet plays an important role in CRC risk.
For instance, high intake of red and processed meats, refined grains and starches and
sugars is related to increased risk for CRC while replacing with poultry, fish, unsaturated
fats, unrefined grains, fruits and legumes is likely to lower CRC risk [25]. Increased
consumption of soy and soy products is associated with reduced risk for CRC; however
which dietary component in soy is responsible for this chemopreventive effect is not well delineated.

Lunasin (Figure 1.1) is a chemopreventive peptide originally isolated from soybean. The word comes from the Tagalog word “lunas” which means cure or treat. It is composed of 43 amino acids with a cell adhesion motif composed of arginine, glycine and aspartic acid situated at residue 33 to 35 followed by a polyaspartic acid region composed of eight aspartic acid residues residing at its carboxylic acid end [26]. The first biological activity of lunasin was discovered in 1999. Galvez and de Lumen [27] showed that transient expression of lunasin in mammalian cells resulted in the inhibition of mitosis of human MCF-7 breast cancer cells, murine hepatoma cells Hepa 1c1c7 and murine embryo fibroblast CEH 10T1/2 cells. Moreover, they showed that lunasin affected spindle fiber formation and kinetochore formation and binds preferentially to histone proteins. The first in vivo study on the chemopreventive property of lunasin showed that exogenous application of synthetic lunasin resulted in reduced tumor incidence and delayed tumor formation in chemically-induced skin tumorigenesis in the SENCAR mouse [26]. Jeong and others first showed that lunasin isolated from soybeans inhibited colony formation induced by the ras oncogene and inhibited core H3 histone acetylation. In addition, other studies have shown that lunasin was able to suppress foci formation in E1A-transfected mouse fibroblast NIH 3T3 cells and induced expression of p21 by 5-fold [28], inhibited lipopolysaccharide-induced inflammation in RAW 264.7 macrophages [29-31], caused a G2/M phase arrest and induced apoptosis in L1210 leukemia cells [32] and prevented breast cancer tumorigenesis in a xenograft mouse model [33]. Another important characteristic of this bioactive compound is its
bioavailability. Bioavailability studies of lunasin in both animals and human showed positive results. Hsieh and others [33] showed that lunasin is bioavailable when orally administered in mouse, present in blood and liver, intact and bioactive. A study in men fed with 50 g soy proteins for 5 days showed that lunasin was present in plasma 30 min and 1 h after soy protein ingestion [34]. Since then, lunasin has been found with the same bioactive properties from other plant sources including rye [35], wheat [36], barley [37, 38], Solanum nigrum [39, 40] and amaranth [41, 42].

![Primary structure of lunasin.](image)

Figure 1.1 Primary structure of lunasin. It is a 43-amino acid peptide containing a cell adhesion motif, arginine-glycine-aspartic acid, (bold) and a polyaspartic acid tail (italics) responsible for its reported biological activities.

However, to date, the potential chemopreventive and chemotherapeutic property of lunasin in CRC is not well understood. Therefore, the overall objective of this research was to evaluate the anticolon cancer potential of lunasin isolated from defatted soybean flour in in vitro and in vivo models. The central hypothesis was that lunasin purified from defatted soybean flour can mediate colorectal carcinogenesis by promoting apoptosis and preventing outgrowth of metastasis.

Aim 1 of this research was to develop a method for isolation and purification of lunasin from defatted soybean flour. A soy extract obtained by centrifugation of a defatted soybean flour-water mixture was loaded onto a diethylaminoethyl (DEAE) anion exchange column and lunasin was eluted by changing the concentration of NaCl. DEAE
anion exchange fractions containing high concentrations of lunasin as determined by enzyme linked immunosorbent assay were pooled, concentrated by ultrafiltration and loaded sequentially onto gel filtration chromatographic columns with molecular weight cut-offs of 25 and 7 kDa. The identity of lunasin was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, reverse phase liquid chromatography (RP-HPLC), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and liquid chromatography tandem mass spectrometry (LC/MS-MS).

Aim 2 of this research was to determine the capability of lunasin purified from defatted soybean flour to cause cytotoxicity and induce apoptosis in different human colon cancer cells. The cytotoxicity of lunasin in different colon cancer cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. The mechanism involved was further investigated by cell cycle and apoptosis analyses.

Aim 3 was to evaluate the potential of lunasin to prevent the outgrowth of metastasis using in vitro and in vivo models. The effect of lunasin on integrin signaling via FAK/ERK/NF-κB cascade in different human colon cancer cells was evaluated in vitro. In addition, the capability of lunasin to inhibit metastasis and potentiate the anti-metastatic effect of the chemotherapeutic drug oxaliplatin was determined using a CRC liver metastasis model in vivo.

In summary, we expect that the results of this research will provide a better understanding on the mechanisms by which lunasin can be used to combat colorectal carcinogenesis.
References


CHAPTER 2
OBJECTIVE AND HYPOTHESES

2.1 Objective

The long term goal of this research is to evaluate the anticolon cancer potential of lunasin purified from defatted soybean flour and to provide the mechanism of action by which lunasin can mediate the process of colorectal carcinogenesis.

2.2 Central hypothesis

Lunasin isolated from defatted soybean flour can mediate colorectal carcinogenesis by promoting apoptosis and preventing outgrowth of metastasis.

2.3 Specific aims

Aim 1. To develop a method of isolation and purification of lunasin from defatted soybean flour. (see Chapter 3)

_Hypothesis:_ Combination of ion-exchange chromatography, ultrafiltration and gel filtration chromatography can isolate and purify lunasin from defatted soybean flour.

1.1 To compare the binding and elution profile of synthetic lunasin and lunasin from defatted soybean flour from two anion-exchange columns, Q-Sepharose Fast Flow (Q-FF) and diethylaminoethyl (DEAE).

1.2 To determine the optimum NaCl concentration needed to elute lunasin from Q-FF and DEAE columns.

1.3 To determine the elution profile of soybean lunasin in two gel chromatographic columns with different molecular weight cut-offs 25 kDa and 7 kDa.

1.4 To characterize lunasin purified from defatted soybean flour.
Aim 2. To determine the cytotoxic effect and potential of lunasin to stimulate apoptosis in colon cancer cells \textit{in vitro}. (See Chapters 4 and 5)

\textit{Hypothesis:} Lunasin isolated from defatted soybean flour can cause cytotoxicity and induce apoptosis in human colon cancer cells via caspase-mediated pathway.

2.1 To determine the cytotoxic effect of lunasin in different human colon cancer cells.

2.2 To investigate the effect of lunasin in cell cycle progression and apoptosis of human colon cancer cells.

2.3 To evaluate the effect of lunasin on the expression of cancer related proteins such as Bax, Bcl-2, cytochrome c, caspase-3, nuclear clusterin, p21 and p27.

2.4 To evaluate the effect of lunasin on the activity of different caspases in human colon cancer cells.

2.5 To investigate the effect of lunasin on the expression of genes associated with cell adhesion and extracellular matrix in human colon cancer cells.

Aim 3. To evaluate the potential of lunasin to prevent the outgrowth of metastasis of colon cancer cells \textit{in vitro and in vivo} models. (See Chapter 6)

\textit{Hypothesis:} Lunasin isolated from defatted soybean flour can prevent the outgrowth of metastasis of human colon cancer cells \textit{in vitro and in vivo} models.

3.1 To determine the potential binding of lunasin with $\alpha_5\beta_1$ integrin in human colon cancer cells.

3.2 To evaluate the effect of lunasin in $\alpha_5\beta_1$ integrin signaling through the FAK/ERK/NF-$\kappa$B cascade in human colon cancer cells.
3.3 To determine the effect of lunasin on the migration of human colon cancer cells.

3.4 To investigate the internalization of lunasin in human colon cancer cells.

3.5 To investigate the effect of lunasin on outgrowth of metastasis of human colon cancer cells in mouse model.

3.6 To determine the potentiating effect of lunasin on the effect of the chemotherapeutic drug, oxaliplatin, on preventing the outgrowth of metastasis of human colon cancer cells in mouse model.
CHAPTER 3

ISOLATION AND PURIFICATION OF LUNASIN FROM DEFATTED
SOYBEAN FLOUR

3.1 Abstract

Lunasin is a chemopreventive peptide present in soybean and other plant sources. The high cost involved in obtaining synthetic lunasin limits its application on chemopreventive and nutritional interventions. The objective of this study was to isolate, purify and characterize lunasin from defatted soybean flour. Isolation and purification was achieved by ion-exchange chromatography, ultrafiltration and size exclusion chromatography. The identity of lunasin was established by Western blot, HPLC, MALDI-TOF and LC/MS-MS. The results showed that lunasin eluted from DEAE anion-exchange column at 0.2-0.3 M NaCl and after 1.5 void volumes from size exclusion chromatography with 25 kDa molecular weight cut-off (MWCO). Fractions from both chromatographic techniques consistently showed three peptides with positive immunoreactivity against lunasin mouse monoclonal antibody corresponding to 5, 8 and 14 kDa. LC/MS-MS analysis of the three immunoreactive peptides showed that 5 and 14 kDa bands contained the lunasin epitope, RGDDDDDD DDD while 8 kDa band showed high homology with 2S soy albumin, a lunasin precursor. Further purification of the mixture using a 7-kDa MWCO size exclusion chromatographic column resulted in the purification of the 5 kDa as confirmed by SDS-PAGE, Western blot and MALDI-TOF profiling. In conclusion, combination of ion-exchange chromatography, ultrafiltration and size exclusion chromatography represents a feasible process for the production of

1This chapter is part of the paper V. P Dia, W. Wang, V. L. Oh, B. O. de Lumen, E. Gonzalez de Mejia, Isolation, purification and characterization of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity, Food Chem. 114 (2009) 108-115. Permission granted by ELSEVIER.
purified lunasin from defatted soybean flour.

3.2 Introduction

Epidemiological data have shown an inverse relationship between soybean consumption and incidence of prostate, colon and breast cancers [1-3]. The consumption of soy has also been specifically linked to a reduction of cardiovascular diseases among other chronic risks [4]. Soybean has nutritional and functional benefits given by its content of biologically active peptides, isoflavones, saponins, essential amino acids, fibers, polyunsaturated fatty acids, vitamins and minerals. The functional health benefits and the method of isolation, preparation and purification of biologically active peptides from soybean have been extensively studied [5]. Biologically active peptides are either naturally occurring or can be derived from soy protein hydrolysates by various methods such as enzyme digestion and fermentation. Some peptides have been found to be bioactive and to exert anticancer, antihypertensive, hypocholesterolemic, antiobesity, antioxidant and immunomodulatory properties [6]. Anticancer proteins and peptides found in soybean include lectin [7], Bowman-Birk inhibitor [8] and the most recently discovered lunasin [9].

Lunasin is a chemopreventive peptide which has been isolated from soybean, barley, wheat and other plant sources [10-14]. It is composed of 43 amino acid residues and contains nine aspartic acid residues on its carboxyl end, a cell adhesion motif composed of arginine-glycine-aspartic acid residues and a predicted helix with structural homology to a conserved region of chromatin binding proteins [10, 12]. Lunasin has been reported to have chemopreventive properties, including suppression of colony formation induced by ras-oncogene and inhibition of core H3-histone acetylation. Earlier studies in
animals showed that lunasin can be absorbed intact and can enter target tissues [15]. Recent studies have shown that the anticancer potential of lunasin can be attributed to its capability to selectively kill cells that are being transformed or are newly transformed by disrupting the dynamic of histone acetylation-deacetylation [10, 11, 13]. In order to better understand the role of lunasin in disease prevention, further in vitro and in vivo studies are needed. However, the high cost involve in obtaining synthetic lunasin limits its application on chemopreventive studies. The objective of this research was to develop a method of isolation, purification and characterization of lunasin from defatted soybean flour.

3.3 Materials and Methods

3.3.1 Materials

Defatted soybean (Glycine max (L.) Merill) flour was obtained from Illinois Center for Soy Foods, University of Illinois Urbana-Champaign. Lunasin mouse monoclonal antibody against the lunasin epitope – EKIMEKIQGRGDDDDD was provided by Dr. Ben O. de Lumen (University of California Berkeley, CA, USA). Chromatographic resins and size exclusion chromatographic column with 7 kDa molecular weight cut-off were purchased from GE Healthcare (Buckinghamshire, UK). All other chemicals were purchased from Sigma unless otherwise specified.

3.3.2 Preparation of Soybean Extract

The extract was prepared by mixing 200 g of defatted soy flour with 1 L of deionized water. The mixture was stirred for 90 min and centrifuged at 12000g for 10 min and the supernatant was pooled. The pooled supernatant was again centrifuged for another 10 min to make sure that any suspended materials were removed, after which it
was filtered using 0.22 µm filter (Millipore, Billireca, MA, USA). The extract was kept at 4 ºC until used for chromatographic separation.

3.3.3 Effect of pH and NaCl on binding and recovery of soy protein, natural soybean lunasin and synthetic lunasin on Q Fast Flow (Q-FF) sepharose resin and diethylaminoethyl (DEAE) resin.

The experiment was conducted following the protocol by Williams and Fresca [16]. To determine the effect of pH on binding of soy protein and synthetic lunasin, defatted soybean flour and synthetic lunasin were separately bound to Q-FF and DEAE resins using different binding pH as follows: pH 5.0, 5.5 and 6.0 were maintained with 20 mM piperazine buffer; pH 6.5 and 7.0 were maintained with 20 mM bis-Tris-propane buffer; and pH 7.5, 8.0 and 8.5 were maintained with 20 mM Tris-HCl buffer. To determine the effect of NaCl on elution of soy protein and synthetic lunasin, defatted soybean flour protein extract and synthetic lunasin were separately bound to Q-FF and DEAE resins in 20 mM Tris-HCl buffer pH 7.5 followed by a stepwise elution with different NaCl concentrations ranging from 0.1 to 1.0 M. To study the effect of NaCl on the elution of soy protein and natural soybean lunasin, soy protein was bound to DEAE resin in 20 mM Tris-HCl pH 7.5 and lunasin was eluted using different NaCl concentrations ranging from 0.1 to 1.0 M. Recovered protein was quantified as mg/mL as determined by A_{280nm} /0.6615 and recovered lunasin was quantified using enzyme linked immunosorbent assay (ELISA) (as described below). Absorbance at 405 nm was used to determine the relative lunasin concentration.

3.3.4 Chromatographic isolation and purification of lunasin from defatted soybean flour
Ion-exchange chromatography (IEC). Based on previous results on the effect of pH, resin type and NaCl concentration, approximately 250 mL of soy extract was loaded in pre-equilibrated XK 50/30 column packed with DEAE resin. Anion exchange separation was carried out using 20 mM Tris-HCl pH 7.5 (buffer A) and 20 mM Tris-HCl + 2 M NaCl pH 7.5 (buffer B). Briefly, soy extract was loaded to the column using buffer A for 75 min at a flow rate of 10 mL/min. Then, the bound protein and lunasin were eluted using the following step gradient: 5% buffer B for 25 min, 10% buffer B for 75 min, 20% buffer B for 100 min and 100% buffer B for 25 min, at a flow rate of 10 mL/min. Fractions were collected every 2 min. Starting from fraction 10, every 3-4 fractions were analyzed for total protein using Biorad Protein DC Assay and lunasin concentration using ELISA. Fractions with high concentration of lunasin were pooled for ultrafiltration, size exclusion chromatography, analyzed using SDS-PAGE, Western blot, HPLC and MALDI-TOF for identification.

Size exclusion chromatography (SEC). Prior to separation by size exclusion chromatography, pooled fractions with lunasin from DEAE ion exchange chromatography were ultrafiltered using Amicon ultrafiltration vessel with YM-3000 membrane (Millipore, molecular weight cut off 3000 Da). Ultrafiltration was carried out using 40 psi helium gas until the volume of the retentate was approximately 10% of the original volume. The retentate was loaded in Superdex 75 Prep Grade size exclusion XK 26/70 column. The column was equilibrated with 0.15 M NaCl in Tris-HCl buffer (0.02 M, pH 7.5) for at least 15 min. Briefly, 15 mL of ultrafiltrate were loaded into the column, and lunasin was eluted with 0.15 M NaCl in Tris-HCl buffer at a flow rate of 4 mL/min. Column fractions were collected every 1 min after one void volume. A total of
30 fractions were collected and each fraction was analyzed for protein concentration and lunasin concentration. Further purification of lunasin was performed using a 7kDa size exclusion chromatography using 20 mM Tris-HCl + 0.15 M NaCl as eluting buffer at a flow rate of 1 mL/min. Fractions with high concentrations of lunasin were analyzed by SDS-PAGE and Western blot.

**3.3.5 Quantification of lunasin by enzyme linked immunosorbent assay (ELISA)**

Selected fractions from IEC and each fraction from SEC were analyzed for lunasin concentration by ELISA following the protocol previously reported [17] with some modifications. Briefly, 100 µL of diluted IEC (1:10000) and SEC fractions (1:5000) were plated on a 96-well plate and stored at 4 °C for at least 14 h. The plate was then washed with 0.01M PBS with 0.05% Tween-20, pH 7.4 using ELX 50 Auto Strip Washer from Biotek Instruments (Winooski, VT, USA). Immediately after that, the plate was blocked by incubating with 300 µL of 5% BSA in TBS-1% Tween-20 for 1 h. The plate was washed and incubated with 50 µL of lunasin mouse monoclonal antibody (1:4000 dilution) for 1 h at room temperature. After incubation and washing, the plate was incubated with 50 µL of antimouse IgG alkaline phosphatase conjugate secondary antibody (1:7000) for 1 h at room temperature. After washing, the color was developed by adding 100 µL of color reagent p-nitrophenyl phosphate to each well. The absorbance was read at 405 nm after 20 min incubation using an ELISA plate reader ELX 808 IU from Biotek Instruments (Winooski, VT). The reaction was stopped by adding 100 µL of 3N NaOH at 25 min and read again at 35 min. Lunasin concentration was quantified using a standard curve from different concentrations of synthetic lunasin (y = 0.0536x - 0.1881, R² = 0.95) and reported as µg lunasin/mL. Primary and secondary antibodies
were diluted with 3% BSA, 1% Tween 20 and 0.05 M TBS buffer. All washings were done with 300 µL of washing solution, six times per well at the lowest dispensing rate (150 µL/well/s) and aspiration rate (5 mm/s) to avoid protein detachment.

3.3.6 Protein Concentration Determination by DC Assay

The protein concentration of IEC and SEC fractions was determined using the Protein DC Microplate Assay Protocol (Biorad Laboratories, Hercules, CA). Briefly, 5 µL of diluted samples (1:50) were plated in a 96-well plate and treated with 25 µL of reagent A and 200 µL of reagent B (Biorad Laboratories, Hercules, CA). The plate was agitated gently and incubated for 15 min at room temperature. After incubation, the absorbance was read at 630 nm. The protein concentration was calculated using BSA standard curve \(y = 0.0002x + 0.0435, R^2 = 0.98\).

3.3.7 Identification of Lunasin

Gel electrophoresis. Fractions with high concentration of lunasin from IEC and SEC were analyzed by SDS-PAGE using PhastGel Gradient 8-25 ready gels (GE Healthcare, Buckinghamshire, UK) in Pharmacia LKB Phast System. Samples (12-20 µg protein) were diluted with tricine sample buffer (Biorad Laboratories, Hercules, CA, USA) with 2% β-mercaptoethanol and boiled for 4 min prior to loading. A pre-stained Kaleidoscope polypeptide standard (Biorad Laboratories, Hercules, CA, USA) was used. Two gels were run simultaneously (250 V, 10 mA), one was fixed with 10% acetic acid and 40% methanol for 30 min, stained with Bio-Safe™ Coomasie G250 (Biorad Laboratories, Hercules, CA, USA) for at least 1 h and destained with 10% acetic acid for 30 min. The destained gel was washed with deionized water and the gel picture was taken with a Kodak Image station 440 CF (Eastman Kodak Company, New Haven, CT, USA). The
other gel was transblotted (20V, 25 mA) onto a polyvinylidene fluoride (PVDF) membrane for Western blot analysis.

Western blot analysis. The PVDF membrane with the transferred protein was blocked with 2% ECL™ Advance blocking agent (GE Healthcare, Buckinghamshire, UK) in Tris buffer saline with 1% Tween 20 (TBST) for 1 h, washed for 5 min three times with fresh changes of 0.1% TBST solution (0.1% Tween 20 in Tris-buffered saline) and incubated with lunasin mouse monoclonal antibody (1:1000 dilution) for 16 h at 4 ºC. After washing, the membrane was incubated with ECL™ antimouse IgG horseradish peroxidase conjugate (1:10000 dilution) for 3 h at room temperature. After washing, the membrane was prepared for detection using chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK) following manufacturer’s instructions. The membrane picture was taken with a Kodak Image station 440 CF (Eastman Kodak Company, New Haven, CT, USA).

High performance liquid chromatographic analysis of lunasin isolated from defatted soybean flour. Beckman Coulter Gold chromatograph system was used for HPLC analysis consisting of a 508 autosampler, programmable solvent, UV detector module and VydaC C4 column. Data acquisition and processing was performed with 32 Karat software for data analysis. Active fractions from IEC were filtered in 0.22 µm membrane before injection. Briefly, 100 µL were injected into an HPLC equipped with a VydaC C4 column (25 cm x 4.6 mm, 10 µm particle size) using diode array detector (215 nm) with mobile phase (5% acetonitrile and 0.08% trifluoroacetic acid, buffer A; and 95% acetonitrile and 0.1% trifluoroacetic acid, buffer B) in linear gradient for 30 min at 1 mL/min. Lunasin was identified by its retention time using a synthetic lunasin standard.
Molecular weight determination by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) peptide mass mapping. Active fractions from IEC and SEC were analyzed by MALDI-TOF using an Applied Biosystems Voyager-DE STR (Foster City, CA, USA) at the protein facility of the University of Illinois for molecular weight peptide mapping. The following parameters were used in the analysis: linear mode of operation, positive polarity and 3000-20000 Da Scanning range. The identity of lunasin was established by comparing the molecular weight of the peak to the MALDI-TOF profile of synthetic lunasin.

Reduction, alkylation and trypsin digestion. Bands with positive immunoreactivity towards lunasin mouse monoclonal antibody from SDS-PAGE gel were reduced, alkylated and digested using the following procedures. Each band was cut and crushed and 100 µL of 25 mM NH_4HCO_3 in 50% acetonitrile were added into 0.5 mL microcentrifuge tube and vortexed for 10 min. This procedure was repeated and the gel pieces were dried to completeness using vacuum evaporator. For reduction, 25 µL of 10 mM DTT in 25 mM NH_4HCO_3 were added to dried gel pieces and incubated at 56 °C with shaking for one hour. The supernatant was removed and 100 µL of 25 mM NH_4HCO_3 were added for rinsing. Alkylation was accomplished by adding 50 µL of 55 mM iodoacetamide in 25 mM NH_4HCO_3. The reaction was carried out in the dark at room temperature. The supernatant was removed and the sample was rinsed with 100 µL of 25 mM NH_4HCO_3 followed by two 100 µL of 50% acetonitrile in 25 mM NH_4HCO_3. The sample was dried to completeness with vacuum. Trypsin digestion was accomplished by adding 25 µL of trypsin solution (12.5 ng trypsin/ µL 25 mM NH_4HCO_3) and was carried out at 37 °C for 4-12 h. The digested peptides were extracted using 100 µL 50%
acetonitrile in 5% formic acid and the sample mixture was sonicated for 10 min. The extraction was repeated twice and the three extracts were pooled and dried. The dried peptides were dissolved in 10 µL of 5% acetonitrile in 0.1% formic acid for LC-MS/MS analysis.

Liquid Chromatography/Mass Spectrometry/Mass Spectrometry. Liquid chromatography was carried out in digested peptides using dC18 Atlantis nanoAcuity column, 75 µm x 150 mm, 3 µm particle size (Waters, Milford MA) using aqueous 0.1% formic acid as solvent A and 50% acetonitrile with 0.1% formic acid as solvent B. A linear gradient from 1 to 90% B was run for 80 min and back to 1% B for 10 min with flow rate maintained at 0.25 µL/min. Mass spectrometric analysis was carried out in Q-Tof API-US nanoAcquity UPLC (Waters, Milford MA) tandem mass spectrometer equipped with electron spray ion source. The Q-tof was operated in positive ion mode. The desolvation and source temperatures were set at 120 and 80 ºC, respectively.

3.3.8 Statistical analysis

Experiments were performed for at least three independent replicates and values were reported as mean ± standard deviation.

3.4 Results and Discussion

3.4.1 Effect of pH and NaCl on binding and recovery of soy protein, natural soybean lunasin and synthetic lunasin on Q Fast Flow (Q-FF) sepharose resin and diethylaminoethyl (DEAE) resin

It was found that pH 7.5 was sufficient enough to cause binding of soy proteins and lunasin on Q-FF sepharose and DEAE resins. At this pH, soy proteins (pI ~ 4.5) were negatively charged and as such capable of interacting with positively charged resins. It
was also found that optimizing binding pH is not an effective way to separate lunasin from other soy proteins due to their similar binding profiles. Q-FF and DEAE resins performed similarly regarding lunasin and soy protein binding properties. Separation of lunasin and other soy proteins can be achieved by salt elution. **Figure 3.1** shows the effect of salt concentration on the elution of soy protein and synthetic lunasin from the two resins. Synthetic lunasin can be eluted with 0.3-0.4 M of NaCl from Q-FF resin (**Figure 3.1A**), and with 0.2-0.3 M NaCl from DEAE resin (**Figure 3.1B**), while higher NaCl concentration is needed to achieve optimal elution of other soy proteins. For better lunasin yield and purity, the use of DEAE column is recommended. To further understand if lunasin naturally present in soy performs the same way as synthetic lunasin, the elution profile of natural soybean lunasin on DEAE resin was monitored by ELISA. **Figure 3.2** shows that naturally extracted soy lunasin was mainly eluted at 0.2 M NaCl, similar to synthetic lunasin. However, there was still a high amount of natural soy lunasin bound to DEAE resin after 0.3 M NaCl elution, indicating the effect of the soy protein matrix on lunasin elution or different binding properties between synthetic and natural soy lunasin.

### 3.4.2 Purification of lunasin by anion exchange chromatography

The extract from the defatted soybean flour was purified in a DEAE anion exchange column. A step gradient of NaCl concentration, 0.10, 0.20, 0.30, 0.40 and 2.0 M NaCl was used to elute bound lunasin and soybean proteins from the anion exchange column. **Figure 3.3A** shows the lunasin concentration of the different fractions collected from the DEAE column and the corresponding salt and protein concentrations. Quantification of lunasin by ELISA showed that the majority of lunasin eluted at 0.2-0.4 M NaCl. This salt
concentration is lower than previously reported by Jeong et al. [12] regarding barley lunasin that eluted of the anion exchange column at 0.7 M NaCl. The difference could be explained by the usage of different ion exchange resin, Biogel resin AG, in that study. Fractions 88 and 104 showed high concentration of lunasin per amount of protein eluted and these fractions were further analyzed by SDS-PAGE and Western blot to determine their protein profiles. Figure 3.3B presents Western blot of different fractions eluted by 0.3 – 0.4 M NaCl. It is apparent that other proteins eluted from the column earlier, while lunasin eluted at the latter part of the separation process. This observation was also supported by high concentration of proteins in earlier anion exchange fractions (fractions 55 to 80), while lower protein concentrations were observed on the latter part of the separation process (fractions 85 to 112) as determined by Protein DC assay. Also, from the Western blot of the same set of anion exchange fractions, three peptides showed positive immunoreactivity with lunasin mouse monoclonal antibody corresponding to 5, 8 and 14 kDa. HPLC analysis of fractions 88 and 104 showed the same peak as with synthetic lunasin (Figure 4A). MALDI-TOF peptide mass mapping supported that these three peptides are predominantly present in the same ion exchange fractions (Figure 3.4B). All fractions containing high concentration of lunasin were pooled, ultrafiltered using an YM 3 kDa membrane and further purified by size exclusion chromatography.

3.4.3 Purification of lunasin by size exclusion chromatography

Retentate from ultrafiltration was further purified by size exclusion chromatography using a Prep Superdex 75 resin with a molecular weight cut-off of 25 kDa. Figure 3.5A shows the lunasin and protein concentrations of the different fractions collected from the size exclusion chromatography after 1 void volume. Each fraction was collected every
minute after 1 void volume. It is evident that lunasin eluted from the size exclusion column after 1.5 void volumes while most of the large molecular weight proteins eluted earlier immediately after the void volume. Fractions showing high concentration of lunasin, as quantified by ELISA, were analyzed by SDS-PAGE and Western blot. Figures 3.5B and 3.5C shows the Coomasie blue staining and Western blot of these fractions. It is apparent that high molecular weight proteins eluted early while lunasin was more prominent in latter fractions. Also, Western blot profile of these fractions also showed three peptides, corresponding to 5, 8 and 14 kDa, showing positive reaction with lunasin mouse monoclonal antibody. Analysis of these fractions using native electrophoresis and nonreducing SDS-PAGE showed the same electrophoretic protein profile suggesting that these three peptides are independent with each other. Further purification of fractions containing high concentration of lunasin using a 7 kDa size filtration chromatography showed that 5 kDa lunasin can be separated from the other two immunoreactive proteins towards mouse monoclonal antibody. Analysis of fractions collected from the 7 kDa size exclusion chromatography showed that fractions 11 to 20 contained appreciable amount of lunasin as determined by ELISA (Figure 3.6A). SDS-PAGE and Western blot analyses (Figures 3.6B and 3.6C) of these fractions showed that fractions 14, 18 and 20 were mainly composed of 14 and 8 kDa, 8 kDa and 5 kDa, respectively. These observations are also supported by MALDI-TOF analysis of these fractions (Figure 3.7).

3.4.4 LC/MS-MS analysis of in-gel tryptic digest of lunasin

To further characterize the identity of the three peptides showing positive reactivity with lunasin mouse monoclonal antibody, an LC/MS-MS analysis of in-gel tryptic digest of
these peptides was performed. The three peptides corresponding to 5, 8 and 14 kDa were cut from SDS-PAGE gel of size exclusion chromatographic fractions. Table 1 summarizes the amino acid sequences found in three peptides and their corresponding monoisotopic masses. LC/MS-MS analysis of the three peptides unambiguously identified the epitope, RGDDDDDD DDD, for lunasin monoclonal antibody in 5 and 14 kDa bands while 8 kDa band showed high homology with 2S soy albumin, a lunasin precursor. The 8 kDa peptide showed nonspecific binding with antimouse IgG horseradish peroxidase conjugate secondary antibody which explains its positive immunoreactivity towards lunasin mouse monoclonal antibody even though it does not contain the epitope for lunasin as shown by LC/MS-MS results. Also, 5 and 14 kDa peptides corresponded to lunasin while the 8 kDa peptide is a 2S soy albumin, a lunasin precursor. The 14 kDa peptide was also found to contain amino acid sequences for Kunitz type inhibitor.

In summary, we found that combination of ion-exchange chromatography, ultrafiltration and size exclusion chromatography is a feasible method for producing purified lunasin from defatted soybean flour.

3.5 References


### Table 3.1 - Peptides in in-gel tryptic digest of three bands showing positive immunoreactivity with lunasin mouse monoclonal antibody and their corresponding isotopic masses.

<table>
<thead>
<tr>
<th>Band</th>
<th>Monoisotopic masses</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 kDa</td>
<td>2179.9001</td>
<td><strong>IQGRGDDDDDDDDDDNHILR</strong></td>
</tr>
<tr>
<td></td>
<td>1310.6653</td>
<td>QLQGVNLTPCEK</td>
</tr>
<tr>
<td></td>
<td>2818.2212</td>
<td>HIMEKI<strong>IQGRGDDDDDDDDDDNHILR</strong></td>
</tr>
<tr>
<td>8 kDa</td>
<td>1459.65</td>
<td><strong>IMENQSEELEEK</strong></td>
</tr>
<tr>
<td></td>
<td>1071.20</td>
<td>C<strong>CTEMSEL</strong>R</td>
</tr>
<tr>
<td></td>
<td>1144.57</td>
<td>E<strong>LINLATMCR</strong></td>
</tr>
<tr>
<td></td>
<td>872.50</td>
<td><strong>GR</strong>IN<strong>YIR</strong></td>
</tr>
<tr>
<td>14 kDa</td>
<td>2179.9001</td>
<td><strong>IQGRGDDDDDDDDDDNHILR</strong></td>
</tr>
<tr>
<td></td>
<td>1310.6653</td>
<td>QLQGVNLTPCEK</td>
</tr>
<tr>
<td></td>
<td>2818.2212</td>
<td>HIMEKI<strong>IQGRGDDDDDDDDDDNHILR</strong></td>
</tr>
</tbody>
</table>

* Bold letters represent the amino acid sequences that matched with 5 kDa lunasin soy peptide (sequence as follows: SKWQHQDSCRKRQLQGVNLTPCEKHIMEKI QGRGDDDDDD DDD). Italics letters represent the amino acid sequences that matched with 2S soy albumin, a lunasin precursor.
Figure 3.1 Effect of elution salt concentration on recovery of soy protein and synthetic lunasin from Q-Fast Flow sepharose resin (A) and DEAE resin (B). The experiments were conducted following the protocol of Williams A and Frasca V. (Williams A and Frasca V. 2001. Ion-exchange chromatography, in Current protocols in protein science, John Wiley & Sons, Inc. New York, NY). Soy protein and synthetic lunasin were bound to resin in 20 mM Tris-HCl buffer pH 7.5 and eluted with different concentration of NaCl. Recovered protein was determined as: protein concentration (mg/mL) = $A_{280\text{nm}}/0.6615$. Figure 3.1 showed lunasin can be separated from soy protein by ion exchange resin. NaCl concentration at 0.3-0.4 M and 0.2-0.3 M were optimized eluting condition for Q-FF resin and DEAE resin, respectively. DEAE resin was recommended for better lunasin yield and purity.
Figure 3.2 Effect of elution salt concentration on the concentration of natural soy lunasin eluted from DEAE resin. Soy protein were bound to resin in 20 mM Tris-HCl buffer pH 7.5 and eluted with different concentration of NaCl. Recovered lunasin was determined by ELISA and Abs at 405 nm was used as a measure of relative lunasin concentration. Figure 3.2 showed that natural soy lunasin was mainly eluted at 0.2 M NaCl, similar to synthetic lunasin. However, different than synthetic lunasin, there were still high amount of natural soy lunasin remained binding to DEAE resin after 0.3 M NaCl elution, indicating the effect of soy protein matrix or different binding properties of synthetic and natural soy lunasin.
Figure 3.3 Purification of lunasin from soy proteins using ion exchange chromatography. Soy protein extract was loaded on XK 50/30 column packed with DEAE resin and eluted with different concentration of NaCl in 20 mM Tris-HCl buffer at 10 mL/min. Lunasin concentration was analyzed by ELISA and protein concentration was determined by protein DC assay. Fractions were collected for further gel filtration fractionation. Figure 3A showed that soy lunasin eluted from the DEAE column at 0.2-0.4 M NaCl. Figure 3B showed that fractions 104 (lanes 1, 3 and 5) and 88 (lanes 2, 4 and 6) with different dilutions (1:10, 1:20 and 1:5, respectively) had three immunoreactive peptides towards lunasin mouse monoclonal antibody with molecular weights of 14, 8 and 5 kDa.
Figure 3.4 HPLC and MALDI-TOF profiles of anion-exchange fractions with high concentrations of lunasin as determined by ELISA and synthetic lunasin. Fractions 88 (Figure 3.4Aa) and 104 (Figure 3.4Ac) were injected into an HPLC equipped with a Vydac C4 column (25 cm x 4.6 mm, 10 µm particle size) using diode array detector (215 nm) with mobile phase (5% acetonitrile and 0.08% trifluoroacetic acid, buffer A; and 95% acetonitrile and 0.1% trifluoroacetic acid, buffer B) in linear gradient for 30 min at 1 mL/min. Lunasin was identified by its retention time (shown by the arrow) using a synthetic lunasin standard (Figure 3.4Ab). Fraction 88 (Figure 3.4B) was analyzed by MALDI-TOF profiling using the following parameters: linear mode of operation, positive polarity and 3000-20000 Da scanning range. The identity of lunasin was established by comparing the molecular weight of the peak to the MALDI-TOF profile of synthetic lunasin (Figure 3C).
Figure 3.5 Lunasin purification with gel filtration chromatography with molecular weight cut-off of 25 kDa. Lunasin enriched fraction of ion exchange column was ultrafiltered and loaded on Superdex 75 gel filtration column (XK 26/70) and eluted with 20 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl at 4 mL/min. Lunasin and protein concentrations were analyzed by ELISA and DC protein assay, respectively. Figure 3.5A showed that lunasin eluted from the column after 1.5 void volumes. Figures 3.5B and 3.5C show the protein profile and Western blot of selected fractions from gel filtration column.
Figure 3.6 Lunasin purification with gel filtration chromatography with molecular weight cut-off of 7 kDa. Lunasin enriched fraction from 25 kDa gel filtration chromatography was loaded in a 7 kDa gel filtration column. Lunasin was eluted using 20 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl at 1 mL/min. Figure 3.6A showed that other proteins eluted approximately after one void volume while lunasin eluted after two column void volumes. Figures 3.6B and 3.6C showed the protein profile and Western blot of selected gel filtration fractions indicating that lunasin can be separated from the other immunoreactive bands by 7 kDa gel filtration chromatography.
Figure 3.7 MALDI-TOF profiles of selected fractions from 7 kDa gel filtration chromatography. Fractions 14 (Figure 3.7A), 18 (Figure 3.7B) and 20 (Figure 3.7C) were analyzed by MALDI-TOF profiling using the following parameters: linear mode of operation, positive polarity and 3000-20000 Da scanning range. Figure 3.7 showed similar molecular weight protein profile with SDS-PAGE in Figure 3.6B.
CHAPTER 4

LUNASIN PROMOTES APOPTOSIS IN HUMAN COLON CANCER CELLS BY MITOCHONDRIAL PATHWAY ACTIVATION AND INDUCTION OF NUCLEAR CLUSTERIN EXPRESSION

4.1 Abstract

Lunasin is a naturally occurring peptide with arginine-glycine-aspartic acid motif associated to its reported biological activity. We aimed to determine the potential of lunasin from soybean to stimulate apoptosis in HT-29 colon cancer cells. Lunasin caused cytotoxicity to HT-29 cells and induced G2/M cell cycle arrest with simultaneous increased in p21 expression. Lunasin induced apoptosis as evidenced by a twofold increase in the percentage of cells undergoing apoptosis, decreased Bcl-2:Bax ratio from 8.5 to 0.4, increased caspase-3 activity by 77% and increased expression of pro-apoptotic nuclear clusterin by fivefold when compared to untreated cells. In conclusion, lunasin stimulated apoptosis in HT-29 cells by activating apoptotic mitochondrial pathways and inducing expression of the pro-apoptotic nuclear clusterin.

4.2 Introduction

Colorectal cancer (CRC) is the third most common cancer in the United States with approximately 146,870 new cases and 49,920 deaths in 2009 [1]. Human CRC is considered to be a consequence of the accumulation of multiple genetic alterations; studies have shown the capability of bioactive food components to modulate the risk of developing CRC [2]. Soybean consumption is associated with reduced risk of developing certain chronic diseases such as cancer. Population studies aimed to investigate the

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association of soybean intake with CRC showed an inverse correlation between CRC risk and soybean products consumption [3, 4]. This inverse relationship can be attributed in part to several bioactive compounds in soybean such as isoflavones [5, 6], saponins [7], and Bowman-Birk inhibitor [8].

Lunasin is a novel chemopreventive peptide originally isolated from soybean and has demonstrated anticancer properties by altering histone acetylation-deacetylation process [9], suppressing lipopolysaccharide-induced inflammation [10, 11] and promoting apoptosis [12]. It features a polyaspartic acid tail that can bind directly to histones thereby affecting proper complex formation leading to mitotic arrest and cell death. Also, a cell adhesion motif composed of arginine-glycine-aspartic acid (RGD) residues is responsible for its cell internalization and attachment to extracellular matrix [13]. Several studies have shown the importance of RGD motif as it is considered to be a target site for integrin interactions thereby playing an important role in drug delivery targeting tumor angiogenesis [14]. Peptides with RGD motif have been used in the study of cell adhesion, migration, growth and differentiation [15]. Moreover, RGD peptides have shown an important role in suppressing inflammation [16] and promoting apoptosis in various cell lines [17, 18]. Also, we have previously shown that lunasin suppressed inflammation by inhibiting NF-κB pathway [11] and promoted apoptosis in L1210 leukemia cells by direct activation of caspase-3 [12]. Apoptosis is a type of programmed cell death characterized by cell and nuclear shrinkage associated to cleavage of cytoskeletal proteins by aspartate-specific proteases [19], chromatin condensation, nuclear fragmentation and the formation of plasma membrane blebs [20]. Acquired resistance toward apoptosis is one of the hallmarks of nearly all types of cancer [21]. However, the role of lunasin in promoting
apoptosis and the mechanism of action by which it mediates apoptosis in colon cancer cells is not yet elucidated.

The objective of this study was to evaluate the potential of lunasin purified from defatted soybean flour to promote apoptosis in HT-29 human colon cancer cells. Moreover, we determined molecular markers associated to lunasin-mediated apoptosis in vitro and a possible interaction between cisplatin and lunasin in inhibiting proliferation of HT-29 colon cancer cells. We showed in this work, for the first time, that lunasin activates the mitochondrial pathway of apoptosis by reducing Bcl-2:Bax ratio and increasing the expression of the proapoptotic nuclear clusterin isoform.

4.3 Materials and methods

4.3.1 Cell lines
Colon cancer cell line HT-29, normal colon fibroblast CCD-33Co, McCoy 5A medium, Eagle's Minimum Essential Medium and 0.25% (w/v) Trypsin- 0.53 mM EDTA were purchased from American Type Culture Collection (Manassas, VA).

4.3.2 Antibodies and reagents
Sodium pyruvate, Annexin V-FITC apoptosis detection kit, penicillin-streptomycin stabilized solution and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Caspase-3 fluorescence assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). Primary antibodies for actin, Bax, Bcl-2, clusterin and p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse IgG and antirabbit IgG horseradish conjugate secondary antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Lunasin (~ 90%) was purified from defatted soybean flour as
we have previously reported [10, 11]. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

4.3.3 Cell proliferation assay

HT-29 colon cancer cells were cultured in McCoy 5A medium containing 10% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate at 37 °C in 5% CO$_2$/95% air. CCD-33Co colon fibroblast was cultured in Eagle's Minimum Essential Medium containing 10% FBS and 1% penicillin/streptomycin. The cell proliferation assay was conducted using the CellTiter 96 Aqueous One Solution Proliferation assay kit containing the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corporation, Madison, WI, USA). For HT-29 cells, 5 x 10$^4$ cells per well were seeded in a 96-well plate and total volume was adjusted to 200 µL with growth medium. The cells were allowed to grow for 24 h at 37 °C in 5% CO$_2$/95% air. After 24 h incubation, the cells were treated with different concentrations of lunasin ranging from 1 to 100 µM, different concentrations of cisplatin ranging from 0.1 to 50 µM and a combination of lunasin (30 and 60 µM) and cisplatin (0.1 to 50 µM) for 24 h. For CCD-33Co, 1 x 10$^3$ cells per well were seeded in a 96-well plate and allowed to grow to confluency for one week with replacement of medium every other day. The cells were then treated with different concentrations of lunasin ranging from 1 to 100 µM for 24 h. After lunasin treatment for both cells, the growth medium was replaced by 100 µL fresh growth medium and 20 µL MTS/PES were added to each well. The plate was incubated for 2 h at 37 °C in 5% CO$_2$/95% air and the absorbance read at 515 nm. The percentage of viable cells was calculated with respect to cells treated with
phosphate buffered saline (PBS) as vehicle. The test was conducted for at least two independent trials with three replicates for each trial.

### 4.3.4 Crystal violet staining

Cells were seeded at a density of $1 \times 10^5$ cells per well in 24-well plate and treated under the same conditions as described above for the cell proliferation assay. After 24 h of lunasin exposure or control condition, the growth medium was aspirated and the cells were washed twice with ice cold-PBS. The cells were then stained with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 2 min at room temperature. The staining solution was removed and the cells were washed twice with ice-cold PBS. The remaining cells adhering into the wells were observed under the microscope and photographed.

### 4.3.5 Cell cycle distribution of HT-29 colon cancer cells

Analysis of cell cycle distribution was performed using flow cytometry as we have previously reported [12]. Briefly, HT-29 cells were seeded at a density of $2 \times 10^5$ cells per well in a 6-well plate and allowed to grow for 24 h at 37 °C in 5% CO$_2$/95% air. The cells were then treated with different concentrations of lunasin ranging from 1 to 50 µM for another 24 h at 37 °C in 5% CO$_2$/95% air. After treatment, cells were fixed overnight with 70% ethanol at 4 °C and stained with propidium iodide solution (0.1% v/v). Cell cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) at excitation wavelength of 488 nm. Fluorescence emission was measured using a 695/40 nm band pass filter. A total of 20,000 events were collected for each sample. The analysis was performed in triplicate.

### 4.3.6 Apoptosis analysis of HT-29 colon cancer cells
The apoptotic status of the HT-29 colon cancer cells was evaluated by determining the presence of phosphatidylserine on the cell membrane using an Annexin V-FITC apoptosis detection kit by flow cytometry. Briefly, 2 x 10^5 cells per well were seeded in a 6-well plate and allowed to grow for 24 h at 37 °C in 5% CO\(_2\)/95% air. The cells were then treated with different concentrations of lunasin ranging from 1 to 50 µM for 5 and 18 h at 37 °C in 5% CO\(_2\)/95% air. After treatment, cells were washed with PBS twice, trypsinized and suspended in binding buffer at a concentration of 1 x 10^6 per mL. Five hundred µL of lunasin-treated and untreated cells were transferred into a plastic test tube and stained with 5 µL Annexin V-FITC and 10 µL propidium iodide at room temperature for 10 min. The cells were analyzed immediately by LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). The analysis was performed in triplicate.

### 4.3.7 Caspase-3 activity analysis

Caspase-3 activity in HT-29 colon cancer cells was analyzed using a caspase-3 fluorescence assay kit that employs a specific caspase-3 substrate, N-AC-DEVD-N’-MC-R110, which upon cleavage by active caspase-3, generates a highly fluorescent product that can be measured using excitation and emission wavelengths of 485 and 535 nm, respectively (Cayman Chemicals, Ann Arbor, Michigan). The specificity of the substrate was validated using the caspase-3 inhibitor, N-Ac-Asp-Glu-Val-Asp-CHO. Briefly, 5 x 10^4 cells per well were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO\(_2\)/95% air. The cells were then treated with different concentrations of lunasin ranging from 1 to 50 µM, different concentrations of cisplatin ranging from 1 to 50 µM and combination of lunasin (50 µM) and cisplatin (1 to 50 µM) for 18 h. After treatment, the plate was centrifuged at 800 x g for 5 min and the culture was removed by aspiration.
Two hundred µL of assay buffer was added to each well and the plate was centrifuged again at 800 x g for 5 min. The supernatant was removed and 100 µL of lysis buffer was added and incubated at room temperature for 30 min with gentle shaking. After 30 min incubation, the plate was centrifuged at 800 x g for 10 min. After which, 90 µL of the supernatant was transferred into a black 96-well plate and 100 µL of substrate was added. The plate was then incubated in the dark at 37 °C for 30 min and the fluorescent intensity was read using a fluorescence plate reader FLx800tbi (Biotek, Winooski, VT). Caspase-3 activity was calculated using a caspase-3 standard curve run at the same time as the samples. The analysis was performed for six independent wells for each concentration and control.

4.3.8 Analysis of Bax, Bcl-2, n- clusterin and p21 expression of HT-29 cells by Western blot

HT-29 cells were seeded at a density of 2 x 10^5 cells per well in a 6-well plate for 24 h at 37 °C in 5% CO₂/95% air. After 24 h incubation, cells were treated with different concentrations of lunasin ranging from 1 to 50 µM for 18 h. After treatment, cells were washed with PBS twice, trypsinized, suspended in lysis buffer composed of 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol and protease inhibitor cocktail (Thermo Scientific, Rockford, IL). The cell suspension was then sonicated and boiled for 5 min. Equal amount of proteins (15 µg) was loaded in 4-20% Tris-HCl ready gels (Biorad Laboratories, Hercules, CA, USA) for protein separation. The separated proteins were transferred to PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 ºC. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min
each) and incubated with actin, Bax, Bcl-2, clusterin or p21 primary antibodies (1:200) at 4 °C overnight. The membrane was washed again and incubated with antimouse IgG horseradish peroxidase conjugate secondary antibody for actin, Bax, Bcl-2, p21, and antirabbit IgG horseradish peroxidase conjugate secondary antibody for n-clusterin for 3-4 h at room temperature. After incubation and repeated washings, the expression of actin, Bax, Bcl-2, n-clusterin and p21 was visualized using chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK). Analyses were done for four replicates.

4.3.9 Statistical Analysis

Data were analyzed using ANOVA. Means were generated and adjusted with Least Significant Difference using Statistical Analysis System software version 9.1. Significant differences were reported at \( P < 0.05 \).

4.4 Results

4.4.1 Lunasin caused inhibition of proliferation on HT-29 colon cancer cells

As shown in Figure 4.1A, lunasin caused a dose-dependent inhibition of proliferation to HT-29 colon cancer cells. At a concentration of 10 µM, lunasin caused a statistically significant reduction on the number of viable HT-29 cells when compared to cells not treated with lunasin, resulting in 19.3% proliferation inhibition. At the highest concentration used (100 µM) lunasin caused a 62.8% inhibition. The concentration that resulted in 50% inhibition \([IC_{50}]\) was 61.7 µM. A change in the morphology of the cells and the number of viable cell were also seen as determined by crystal violet staining (Figure 4.1B).

Lunasin and cisplatin synergistically inhibited the proliferation of HT-29 colon cancer cells. As shown in Figure 4.1C, cisplatin caused a dose-response reduction in the...
viability of HT-29 colon cancer cells with an IC$_{50}$ of 76.7 µM. In the presence of lunasin (60 µM), the potency of cisplatin (50 µM) resulted in a significant increase in the inhibition of HT-29 colon cancer cell growth. At this combination, the percentage of cell inhibition amounted to 98.6% suggesting a synergistic interaction between the two compounds.

On the other hand, lunasin did not cause any cytotoxicity to CCD-33Co normal colon fibroblast up to 100 µM.

4.4.2 Lunasin caused a G2/M cell cycle arrest on HT-29 colon cancer cells

Figure 4.2A shows the effect of lunasin on HT-29 cell cycle progression. It can be seen that treatment of HT-29 colon cancer cells with lunasin resulted in a dose-dependent enhancement of G2 cell cycle arrest. The amount of cells in the G2 phase increased from 10.3 % (untreated cells) to 19.1%, 20.3% and 26.3% for HT-29 colon cancer cells treated with 1, 10 and 50 µM lunasin, respectively. This also resulted in the significant reduction of cells in the S-phase. To understand the mechanism by which lunasin caused a G2/M cell cycle arrest, we measured the expression of cyclin dependent kinase inhibitor p21 on cell lysates of HT-29 treated with different concentrations of lunasin. Lunasin caused a dose-dependent increase in the expression of p21 (Figure 4.2B). The expression of p21 increased from 7.1% (untreated cells) to 25.7%, 33.3% and 51.6% when HT-29 colon cancer cells were treated with 1, 10 and 50 µM lunasin, respectively.

4.4.3 Lunasin promoted apoptosis on HT-29 colon cancer cells

To determine whether the reduction in cell proliferation of HT-29 colon cancer cells was due to apoptosis, we treated HT-29 colon cancer cells with different concentrations of lunasin for different lengths of time. Treatment of lunasin for 5 h did not result in a
significant increase in the amount of HT-29 cells undergoing apoptosis. At 18 h treatment, lunasin at a concentration of 10 μM caused a significant increase in the amount of apoptotic cells (Figure 4.3). The percentage of apoptotic cells increased from 10.8% (untreated cells) to 14.6% and 18.7% for HT-29 cells treated with 10 and 50 μM lunasin, respectively. Apoptosis was measured at 10 and 50 μM, lower concentrations than the IC₅₀ (61.7 μM) in order to observe the apoptotic event rather than cell necrosis.

4.4.4 Lunasin activated mitochondrial pathway of apoptosis by modifying expression of Bcl-2 proteins on HT-29 colon cancer cells

To further elucidate the mechanism involved in lunasin-mediated apoptosis, we measured the expression of Bcl-2 family of proteins which is associated with the outer mitochondrial membrane. Treatment of HT-29 cells with lunasin resulted in a dose-dependent increase in the expression of pro-apoptotic Bax protein. The Bax protein expression increases from 8.4% (untreated cells) to 23.3%, 55.4% and 60.4% upon treatment of lunasin at 1, 10 and 50 μM, respectively, in HT-29 colon cancer cells (Figure 4.4A). On the other hand, the expression of the anti-apoptotic Bcl-2 protein was dose-dependently reduced by lunasin treatment. Significant inhibition of Bcl-2 expression started at 10 μM lunasin with 35.1% inhibition and went up to 65.7% inhibition at 50 μM lunasin (Figure 4.4B).

4.4.5 Lunasin increased caspase-3 activity in HT-29 colon cancer cells

Caspases are enzymes that belong to the cysteine family that cleave their substrates at aspartic acid residues and play a key role in apoptosis [22]. We examined the effect of lunasin treatment on the activity of one of the caspases involved in executing the apoptotic process, the caspase-3 [23]. Figure 4.5A presents the effect of lunasin on the
caspase-3 activity of HT-29 colon cancer cells. When untreated, the caspase-3 activity of HT-29 colon cancer cell was 175 mU/mL. This activity increased to 258 mU/mL, 286 mU/mL and 310 mU/mL when HT-29 colon cancer cells were treated with 1, 10 and 50 µM lunasin. This corresponds to statistically significant increase in caspase-3 activity by 48%, 63% and 77%, respectively (\(P < 0.05\)). Caspase-3 activity of HT-29 colon cancer cells when treated with 50 µM cisplatin alone was 1137 mU/mL, upon addition of 50 µM lunasin the caspase-3 activity went up to 2220 mU/mL (Figure 4.5B). This observation showed the capability of lunasin to potentiate the effect of a chemotherapeutic drug in promoting apoptosis.

We then measured the expression of nuclear clusterin in HT-29 cell lysates to determine if this pro-apoptotic protein was involved in lunasin-mediated apoptosis. At 10 µM, lunasin caused a significant increased in the expression of nuclear clusterin from 8% (untreated cells) to 44% indicating an approximately fivefold increase in the expression of this pro-apoptotic protein (Figure 4.5C).

4.5 Discussion

Lunasin contains a RGD motif responsible for its cell internalization and interaction with the extracellular matrix. Several studies have shown the importance of the RGD motif as it is well-known for recognizing and interacting with integrin [24]. Moreover, RGD motif together with integrin receptors constitutes a major recognition system for cell adhesion [25] influencing and regulating cell migration, growth, differentiation and apoptosis [26]. In this study we showed for the first time the capability of lunasin to cause decrease in proliferation (Figure 4.1A) and to promote apoptosis in HT-29 colon cancer cells (Figure 4.3). Our previous study showed the capability of lunasin to cause cytotoxicity to
L1210 leukemia cell lines with an IC$_{50}$ of 16 µM [12], a dose four times more effective than what we observed in the case of HT-29 colon cancer cells. These results showed that the cytotoxic activity of lunasin purified from soybean is dependent on the type of cell line treated. Previous studies have shown lunasin not to cause any cytotoxicity to HL60 leukemia and HepG2 hepatoma cells up to a concentration of 3 mg/mL lunasin-enriched soy flour [12] and to human breast cancer cell line MCF-7 and human lung cancer cell line NCI-H460 up to a concentration of 10 µM [27]. Other peptides containing the RGD motif have also shown capability to cause growth inhibition in established cell lines. Buckley et al. [28] showed that RGD peptides caused reduction in cell survival of lymphocytes and leukocytes. This growth inhibitory property was abrogated when the RGD motif was substituted with arginine-alanine-aspartic acid (RAD) motif. When HL60 cells were exposed to RGD-containing peptides at a concentration of 1 mM caused 50% death after 36 h of exposure; this cytotoxic effect was not seen when the RGD motif was substituted with RAD [17]. Matsuki et al. [18] found similar results to Anuradha et al. [17] in chondrocytes and synovial cell lines. Our results together with the results of previous studies showed the importance of the RGD motif in causing cytotoxicity to various cell lines. We further analyzed a possibility of having a synergistic interaction between lunasin and a known chemotherapeutic drug, cisplatin. Combined treatment of lunasin and cisplatin resulted in a synergistic growth inhibition of HT-29 colon cancer cells when lunasin was added at 30 and 60 µM in different concentrations of cisplatin. The results of cell cycle distribution showed the capability of lunasin to inhibit cells to enter the mitosis stage which is important for cells with damaged DNA [29]. Our study in L1210 leukemia cells also showed the capability of lunasin to arrest cell cycle at the G2
stage [12]. Several studies also showed the capability of RGD to affect cell cycle distribution in cancer cells. Chen et al. [30] showed that bicyclic RGD peptide resulted in a G0/G1-phase arrest in MDA-MB-435 metastatic breast cancer cells while conjugation of this peptide with paclitaxel, an antimicrotubule agent, resulted in G2/M-phase arrest. The same G1-phase result was demonstrated in breast cancer cells MDA-MB-231 and MCF-7 when treated with 10-amino acid peptide containing the RGD sequence in a cyclic conformation [31]. The differences of the previous studies and our study might be attributed to the nature of the peptides used. In the studies resulting in G1-phase arrest, cyclic RGD peptides were used while our experiments used a purified naturally-occurring RGD-containing peptide, lunasin (Figure 4.2A). Moreover, lunasin contains the polyaspartic acid tail which has been reported to directly bind histone thereby affecting proper complex formation leading to mitotic arrest, which is what we observed in this study. The G2/M-phase of cell cycle is being controlled by one master regulatory kinase called cyclin-dependent kinase 1 (CDK1) and can be arrested by various stress stimuli including down regulation of CDK1 and topoisomerase IIα expression and upregulation of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 [32]. We then measured the expression of CDK inhibitor p21 in HT-29 colon cancer cells to further investigate the reason for G2/M-phase arrest mediated by lunasin (Figure 4.2B). Our results demonstrated the capability of lunasin to enhance expression of p21 in HT-29 colon cancer cells by sevenfold. CDK inhibitor p21 can block the phosphorylation of human CDK1, cell division cycle 2, at threonine 161 thus arresting cell cycle at G2-phase [33]. In addition, HCT-116 colon cancer cells lacking p21 resulted in a transient and defective
G2-phase arrest [34]. The increased in p21 expression of HT-29 colon cancer cells when treated with lunasin explains the G2/M-phase arrest caused by lunasin treatment. Apoptosis is a naturally occurring phenomenon that is genetically controlled and it is important in normal development and maintenance of tissue homeostasis [35]. Lunasin treatment resulted in a significant increase in the percentage of HT-29 cells undergoing apoptosis (Figure 4.3). The increase in the percentage of apoptotic cells was accompanied by the modification of the expression of Bcl-2 family of proteins, Bax and Bcl-2. The Bcl-2 family of proteins is important in mediating the intrinsic or mitochondrial pathway of apoptosis (Figure 4.4). Bcl-2 is a known anti-apoptotic protein that is frequently examined for potential clinical use as a prognostic biomarker in cancer [36] and its over-expression is associated with resistance to cytotoxic drugs such as cisplatin and 5-fluorouracil [5-FU] [37 - 38]. Also, studies have shown that aberrant expression of this protein facilitates tumor progression in the early stages of colon cancer [39, 40]. Our results showed that lunasin treatment resulted in a dose-dependent reduction in the expression of Bcl-2 proteins in HT-29 colon cancer cells with 65.7% down regulation of Bcl-2 expression at 50 µM. This reduction on the expression of the anti-apoptotic Bcl-2 is concomitant with increased expression of the pro-apoptotic Bax protein. Lunasin at 50 µM resulted in a sevenfold increase in the expression of Bax by HT-29 colon cancer cells. Bax is essential in mitochondrial mediated apoptosis as its insertion in the mitochondrial membrane resulted in the release of cytochrome c into the cytosol leading to the activation of caspases thereby committing the cells to apoptosis [41, 42]. In CRC, Bax positively correlated with better survival in advanced metastasis [43, 44]. Our results showed that lunasin mediated apoptosis through modification of Bcl-
2 family of proteins involved in apoptotic mitochondrial pathway. Moreover, we showed here for the first time the capability of lunasin to alter Bcl-2:Bax ratio in such a way that the apoptotic pathway will be triggered in HT-29 colon cancer cells in culture. Our results showed that lunasin treatment caused an increase in the activity of caspase-3 in HT-29 colon cancer cells (Figure 4.5A). This observation may be attributed to the RGD motif of lunasin and further supported our previous study on the direct activation of caspase-3 by lunasin in L1210 leukemia cell lines [12]. Moreover, several studies have shown the capability of RGD-containing peptides to stimulate apoptosis in different cancer cell lines. Anuhadra et al. [17] showed that RGD-treated HL60 leukemia cells resulted in caspase-3 activation thereby inducing apoptosis in HL60 cells. RGD-Fas L demonstrated capability to induce apoptosis in different pituitary adenoma cells through caspase activation as evidenced by increased expression of caspases-3, -8 and -9 and decreased expression of Bcl-2 [24]. Also, it has been shown that at 10 mM YGRGD (tyrosine-glycine-arginine-glycine-aspartic acid) peptide resulted in induction of apoptosis in adenocarcinoma lung cancer cells A549 and murine mammary cancer cells EMT-6 [45]. The same result was found by Yang et al. [31] when breast cancer cell lines MDA-MB-231 and MCF-7 were treated with 10-amino acid RGD-containing peptide. Moreover, a synergistic interaction in increasing caspase-3 activity was also observed when lunasin (50 µM) was added at different concentrations of cisplatin in HT-29 colon cancer cells (Figure 4.5B). The activity of caspase-3 became significantly higher than expected additive effect (P < 0.05). These results for the first time showed a possible use of lunasin-drug combination in mitigating cancer which is important in order for chemotherapeutic drugs to be used in lower concentrations resulting in less side effects
and cytotoxicity to normal cells. We have shown that lunasin did not affect the growth of normal colon fibroblasts.

We further measured the effect of lunasin on the expression of a newly suggested potential biomarker for CRC called clusterin. Clusterin is an apolipoprotein with ubiquitous distribution and is implicated in several diverse physiological processes [46]. Furthermore, its expression is associated with contradictory functions either tumor progression, resistance to treatment in vivo, cell survival or apoptosis associated to its different isoforms [47, 48]. Our results showed that lunasin treatment induced the expression of the nuclear clusterin isoform (nCLU) in a dose-dependent manner. As for our knowledge, this is the first report on the induction of nCLU in colon cancer cells by a dietary compound. A study on the same HT-29 colon cancer cell line showed that addition of 5-FU and anti-Fas antibody resulted in increase mRNA and protein expression of nCLU correlating with the increase in the percentage of cells undergoing apoptosis [49]. Also, the same study showed that the induction of apoptosis by nCLU expression is dependent of p21 expression as homozygous p21 knockout HCT-116 cell line did not undergo apoptosis nor expressed nCLU upon treatment of 5-FU and anti-Fas antibody. Our results showed that when HT-29 colon cancer cells were treated with lunasin, nCLU expression was induced which might be associated with simultaneous increase in p21 expression. This also shows that aside from activation of the mitochondrial pathway through increase caspase-3 activity, lunasin also mediate apoptosis through increased expression of p21 resulting in the induction of nCLU expression, a pro-apoptotic isoform of clusterin. It has recently been shown that apoptosis induced by cisplatin on human cancer cells depends on the mitochondrial serine protease
Omi/Htra2 that is released into the cytosol drying apoptosis to antagonize inhibitors of apoptosis and contribute to caspase-independent cell death [50]. Omi/Hrta2 is involved in the intrinsic/mitochondrial pathway of apoptosis; however, the present study does not allow to determine if lunasin may also work on this specific pathway. It is also important to learn if lunasin-induced-apoptosis can prevent migration and invasion of cancer cells. This, however, needs to be the subject of a separate study. Our in vivo human study on the bioavailability of lunasin [51] showed the presence of lunasin in the plasma of young, healthy men after consumption of 50 g soy proteins for 5 days. This supports the idea that lunasin can be used to aid patients suffering from cisplatin-nephrotoxicity. Giving lunasin as a supplement would allow the use of cisplatin at a lower concentration thus reducing its toxic effect to the kidneys. Clinical studies are needed to test this hypothesis.

In summary, we propose a model by which lunasin promoted apoptosis in HT-29 colon cancer cells in vitro through activation of mitochondrial pathway as evidenced by increased expression of Bax, decreased expression of Bcl-2 and increase in the activity of caspase-3. Also, a synergistic interaction between cisplatin and lunasin was observed in inhibiting HT-29 colon cancer cell proliferation and promoting caspase-3 activity. Moreover, lunasin induced the expression of the pro-apoptotic nCLU which might be associated with simultaneous increase in p21 expression.

4.6 References


Figure 4.1 Lunasin caused a dose-dependent reduction in proliferation of HT-29 colon cancer cells. A. HT-29 colon cancer cells were seeded and allowed to grow for 24 h and treated with lunasin at various concentrations for another 24 h. The amount of live cells were then determined using MTS assay. B. HT-29 colon cancer cells were treated with different concentrations of lunasin for 24 h, stained with crystal violet, observed and photographed under the microscope. C. Synergistic cytotoxic effect in HT-29 colon cancer cells by lunasin and cisplatin combination. HT-29 colon cancer cells were seeded and allowed to grow for 24 h after which cisplatin was added at various concentration (1 to 50 µM) in the presence or absence of lunasin (30 and 60 µM) for another 24 h. The amount of live cells were then determined using MTS assay. Means within cisplatin concentration followed by * sign is different from cisplatin treatment alone showing a synergistic interaction between cisplatin and lunasin (n = 6, P < 0.05).
Figure 4.2 Lunasin arrested cell cycle progression at G2/M-phase and increased the expression of cyclin dependent kinase inhibitor p21. A. Cell cycle progression analysis of cells as affected by lunasin treatment was performed by flow cytometry using propidium iodide staining. B. p21 expression of HT-29 colon cancer cells as affected by lunasin treatment was determined using Western blot. Means with different letters are significantly different from each other (n= 3). An example of Western blot for p21 expression is presented as an inset from four different replicates with similar results, $P < 0.05$.
Figure 4.3 Lunasin induced apoptosis of HT-29 colon cancer cells. Apoptosis was measured after treatment of confluent cells for 18 h with different concentrations of lunasin (1 to 100 µM), after which cells were washed and suspended in binding buffer and stained with propidium iodide and Annexin V. Means with different letters are significantly different from each other (n= 3, \( P < 0.05 \)).
Figure 4.4 Lunasin activated mitochondrial pathway of apoptosis by increasing Bax and reducing Bcl-2 expression of HT-29 colon cancer cells. A. Bax expression. B. Bcl-2 expression as determined by Western blot. Means with different letters are significantly different from each other (n= 4) ($P < 0.05$). An example of Western blot is presented as an inset from four different replicates with similar results.
Figure 4.5 Lunasin increased caspase-3 activity and nuclear clusterin (nCLU) expression of HT-29 colon cancer cells. A. Caspase-3 activity was determined in HT-29 colon cancer cells after 18 h of treatment with lunasin (1 to 50 µM) using a fluorescence assay kit. B. Activation of caspase-3 by lunasin and cisplatin combination. Caspase-3 activity was determined in HT-29 colon cancer cells after 18 h of treatment with cisplatin (1 to 50 µM) in the presence or absence of lunasin (50 µM) using a fluorescence assay kit. Means with different letter are significantly different from each other (n=6); means within cisplatin concentration followed by a different symbol are significantly different from cisplatin treatment alone showing a synergistic interaction between cisplatin and lunasin (n=6). C. Nuclear clusterin expression was measured using Western blot. Means with different letters are significantly different from each other (n = 3, $P < 0.05$). An example of Western blot is presented as an inset from three different replicates with similar results.
CHAPTER 5
LUNASIN PROMOTES APOPTOSIS AND MODIFIES GENES ASSOCIATED WITH EXTRACELLULAR MATRIX AND CELL ADHESION IN HUMAN METASTATIC COLON CANCER CELLS³

5.1 Abstract

The potential of the peptide lunasin to induce apoptosis in human colon cancer cells and their oxaliplatin-resistant (OxR) variants was evaluated. Also its effect on the expression of human extracellular matrix and adhesion genes was determined. Various human colon cancer cell lines, which underwent metastasis following a mouse model, were evaluated in vitro using cell flow cytometry and fluorescence microscopy. Lunasin cytotoxicity to different colon cancer cells correlated with the expression of $\alpha_5\beta_1$ integrin; being most potent to KM12L4 cells ($IC_{50}$ of 13 µM). Lunasin arrested cell cycle at G2/M phase with concomitant increase in the expression of cyclin dependent kinase inhibitors p21 and p27. Lunasin (5-25 µM) activated the mitochondrial pathway of apoptosis as evidenced by changes in the expressions of Bcl-2, Bax, nCLU, cytochrome c and caspase-3 in KM12L4 and KM12L4-OxR. Lunasin increased the activity of initiator caspase-9 leading to activation of caspase-3, and also modified the expression of human extracellular matrix and adhesion genes; downregulating integrin $\alpha_5$, SELE, MMP10, integrin $\beta_2$ and COL6A1 by 5.01-, 6.53-, 7.71-, 8.19- and 10.10-fold, respectively while upregulating COL12A1 by 11.61-fold. The results suggest that lunasin can be used in cases where resistance to chemotherapy developed.

³This chapter is a pre-peer reviewed version of the article V.P. Dia, E. Gonzalez de Mejia, Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. Mol. Nutr. Food Res. 55 (2011) 623-634. Permission granted by Wiley-VCH
Lunasin is a chemopreventive peptide originally isolated from soybean [1]. It is also found in other plants such as amaranth [2], barley [3], Solanum [4] and wheat [5]. Its chemopreventive and anticancer properties are demonstrated by suppressing lipopolysaccharide-induced inflammation [6 – 8] and inducing apoptosis in different cancer cell lines [9 -11]. These properties are associated with unique amino acid sequences present in lunasin which included a cell adhesion motif composed of arginine-glycine-aspartic acid (RGD) residues and a polyaspartic acid tail composed of nine aspartic acid residues. The RGD motif is responsible for the internalization of lunasin into the cells and the polyaspartic acid tail affects kinetochore formation leading to mitotic arrest [1, 12]. Previous studies have also demonstrated the capability of RGD-containing peptides to promote apoptosis to different cell lines [13, 14]. Apoptosis is a natural phenomenon important in maintenance of homeostasis and normal development. It is characterized by a series of morphological changes including loss of specialized surface structure, reduction in volume, conservation of cytoplasmic organelles, condensation of nuclear chromatin and phagocytosis [15, 16]. Acquired resistance towards apoptosis is a hallmark of almost all types of cancer cells [17].

Colorectal cancer [CRC] is the third most common cancer in the U. S. with estimated 50,000 new cases occurring every year [18]. Its management cost for 2020 has been estimated in $14 billion [19]. Although significant progress has been made in the treatment of CRC with the use of adjuvant therapies, the 5-year survival success rate for patients with metastatic CRC is still 8% [20]. This poor survival rate for patients with metastatic CRC is, in part, attributed to development of resistance to chemotherapy. For
instance, resistance to oxaliplatin, a commonly used chemotherapeutic drug for patients with metastatic CRC, is associated with altered mitochondrial-mediated apoptosis as evidenced by complete loss of pro-apoptotic Bax and undetectable expression of active caspase-3 [21]. Moreover, acquired resistance to oxaliplatin is associated with independent defects in both drug uptake by cells as well as in formation of DNA adducts [22]. In addition to resistance-development, use of chemotherapeutic drugs has been associated with adverse side effects including acute and chronic neuropathy, hypersensitivity reactions, diarrhea, neutropenia and hand-foot syndrome [23]. As such, it is important to find naturally occurring compounds that can enhance the effectiveness of chemotherapeutic drugs thereby preventing resistance-development.

The objective of this study was to evaluate the potential of lunasin to promote apoptosis in human CRC cells and in their oxaliplatin-resistant (OxR) variants. Moreover, we assessed the effect of lunasin on the expression of human extracellular matrix (ECM) and adhesion genes in KM12L4 cells. We showed for the first time, the capability of lunasin to induce apoptosis in KM12L4 and KM12L4OxR human metastatic CRC cells. This effect might be attributed to its capability to modify the expression of human ECM and adhesion genes suggesting the potential use of lunasin as an adjuvant to currently used chemotherapy for patients with metastatic CRC, with the advantage of reduced cytotoxicity.

5.3 Materials and methods

5.3.1 Cell lines

HCT-116, HT-29, KM12L4, RKO cells and their OxR variants designated as HCT-116OxR, HT-29OxR, KM12L4OxR and RKOOxR were obtained from Dr. Lee M. Ellis
(MD Anderson Cancer Center, University of Texas). The metastatic KM12L4 cell line was established by injecting the parental cell line KM12C into the spleen of nude mice as previously reported [24]. The OxR variants were prepared by repeated exposure of the parental cell lines to increasing concentration of oxaliplatin as reported earlier [25]. The normal human colon fibroblasts CCD-33Co were purchased from American Type Culture Collection (Manassas, VA).

5.3.2 Cell proliferation

Cells were cultured in minimum essential medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, 1% vitamin solution and 1% sodium pyruvate at 37 °C in 5% CO\textsubscript{2}/95% air. CCD-33Co colon fibroblasts were cultured in Eagle's Minimum Essential Medium containing 10% FBS and 1% penicillin/streptomycin. The cell proliferation assay was conducted using the CellTiter 96 Aqueous One Solution Proliferation assay kit (Promega Corporation, Madison, WI, USA). For parental cells, 5 x 10\textsuperscript{3} cells per well were seeded in a 96-well plate while 2.5 x 10\textsuperscript{3} cells per well were seeded for oxaliplatin-resistant variants and total volume was adjusted to 200 µL with growth medium. The cells were incubated for 24 h at 37 °C in 5% CO\textsubscript{2}/95% air. After 24 h incubation, the cells were treated for another 24 h with different concentrations of lunasin purified (>90%) from defatted soybean flour as reported previously [6]. For CCD-33Co, 1 x 10\textsuperscript{3} cells per well were seeded in a 96-well plate and allowed to grow to confluency for one week with replacement of medium every other day. The cells were then treated with different concentrations of lunasin for 24 h (0 to 100 µM). After lunasin treatment, cell viability was determined by MTS assay as previously reported [6]. An interaction study between lunasin (10 and 25 µM) and
oxaliplatin (0.5, 1.0 and 2.0 µM) was performed for KM12L4 colon cancer cells following the same cell proliferation protocol described above. For OxR cells, 2 µM oxaliplatin (final concentration) was added throughout treatment. Each test was conducted for at least two independent trials with three replicates for each trial.

5.3.3 Cell cycle distribution of KM12L4 colon cancer cells

Analysis of cell cycle distribution was performed using flow cytometry as we have previously reported [9]. Briefly, KM12L4 cells were seeded at a density of 2 x 10^5 cells per well in a 6-well plate and allowed to grow for 48 h at 37 °C in 5% CO₂/95% air. Cells were then treated with different concentrations of lunasin (0 to 10 µM) for another 24 h at 37 °C in 5% CO₂/95% air. After treatment, cells were fixed overnight with 70% ethanol at 4 °C and stained with PI solution. Cell cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) at excitation wavelength of 488 nm. Fluorescence emission was measured using a 695/40 nm band pass filter. The analysis was performed in triplicates.

5.3.4 Apoptosis of KM12L4 colon cancer cells

Apoptosis of KM12L4 colon cancer cells was evaluated by flow cytometry using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St Louis, MO). Briefly, 2 x 10^5 cells per well were seeded in a 6-well plate and allowed to grow for 48 h at 37 °C in 5% CO₂/95% air. The cells were then treated with different concentrations of lunasin (0 to 25 µM) for 24 h at 37 °C in 5% CO₂/95% air. After treatment, cells were washed with PBS twice, trypsinized and suspended in binding buffer at a concentration of 1 x 10^6 per mL. Five hundred µL of lunasin-treated and untreated cells were transferred into a plastic test tube and stained with 5 µL Annexin V-FITC and 10 µL PI solution for 10 min. The cells
were analyzed immediately by LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). The analysis was performed in triplicate.

5.3.5 Fluorescence microscopy of KM12L4 colon cancer cells

Fluorescence microscopic analysis of cells was performed at the Institute of Genomic Biology Microscopy Facility University of Illinois at Urbana-Champaign. Briefly, $1 \times 10^3$ cells suspended in 300 µL medium were seeded in an 8-well ibiTreat plate (ibidi Integrated BioDiagnostics, Martinsried, Germany) allowed to grow to 60-70% confluence at $37 \, ^\circ \mathrm{C}$ in 5% CO$_2$/95% air. Cells were treated with 10 and 25 µM lunasin for 24 h. After treatment, cells were washed and fixed with 4% paraformaldehyde in PBS for 30 min. The cells were washed with PBS three times and permeabilized with 0.1% Triton-X for 30 min and washed again with PBS. The cells were stained with Hoechst 33342 stain (Invitrogen, CA) and mounted with prolong gold (Invitrogen, CA) for 24 h in the dark at room temperature. The plate was kept at 4 °C in the dark until analysis. The cells were imaged using Zeiss Axiovert 200M with the Apotome Structured Illumination Optical Sectioning System fluorescence microscope (Zeiss Obercohen, Germany).

5.3.6 Analysis of protein expression on KM12L4 and KM12L4OxR cells by Western blot

KM12L4 and KM12L4OxR cells were seeded at a density of $2 \times 10^5$ and $1 \times 10^5$ cells per well, respectively, in a 6-well plate for 48h at $37 \, ^\circ \mathrm{C}$ in 5% CO$_2$/95% air. In case of KM12L4OxR cells, 2 µM oxaliplatin (final concentration) was added throughout treatment. Cells were then treated with different concentrations of lunasin (0 to 25 µM) for 24 h. Cells were washed with PBS twice, trypsinized, suspended in lysis buffer composed of 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol
blue, 5% β-mercaptoethanol and protease inhibitor cocktail (Thermo Scientific, Rockford, IL). The cell suspension was sonicated and boiled for 5 min. Equal amount of protein (approximately 15 µg) was loaded in 4-20% Tris-HCl ready gels (Biorad Laboratories, Hercules, CA, USA). The separated proteins were transferred to PVDF membrane and blocked with 5% non-fat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 ºC. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min each) and incubated with actin, Bax, Bcl-2, clusterin, cytochrome c, integrins α₅ and β₁, p27, or p21 primary antibodies (1:200) (Santa Cruz Biotechnology, CA) at 4 ºC overnight. The membrane was washed again and incubated with antimouse IgG horseradish peroxidase conjugate secondary antibody for actin, Bax, Bcl-2, cytochrome c, caspase-3, integrins α₅ and β₁, p27 and p21 (GE Healthcare, Buckinghamshire, UK) and antirabbit IgG horseradish peroxidase conjugate secondary antibody for clusterin (GE Healthcare, Buckinghamshire, UK) for 3-4 h at room temperature. After incubation and repeated washings, the expression of proteins was visualized using chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK). Analyses were conducted in triplicate.

**5.3.7 Caspase activity**

The activity of caspases-2, -3, -6, -8, and -9 was determined using the caspase colorimetric assay kit (Invitrogen, CA) following manufacturer’s protocol. Briefly, cells were seeded at a density of 2 x 10⁵ per well in a 6-well plate and allowed to grow for 48 h at 37 ºC in 5% CO₂/95% air. Cells were treated with different concentrations of lunasin (0 to 10 µM) for 24 h. After treatment, cells were pelleted and resuspended in 50 µL chilled cell lysis buffer and incubated on ice for 10 min. The cells were then centrifuged
at 10,000 x g for 1 min. Approximately 50 µg protein was assayed for caspase activity analysis by adding 50 µL reaction buffer containing 10 mM dithiothreitol and 5 µL of the 4 mM corresponding caspase substrates in a 96-well plate. The plate was incubated at 37 °C in the dark for 2 h. The absorbance was read at 405 nm using ELx808 microplate reader (Biotek, Winooski, VT). Analyses were performed in four replicates.

5.3.8 Extracellular matrix (ECM) gene expression profile using real time polymerase chain reaction

KM12L4 cells were plated at a density of 2 x 10^6 cells in a 75 cm^2 flasks, cultured overnight and treated with 10 µM lunasin or PBS for 24 h. Cells were harvested by trypsinization and total RNA was harvested using RNeasy Mini Kit with an on-column DNase treatment step (Qiagen, CA). Four hundred nanogram of RNA from each sample was used to carry out reverse transcription using a RT² First Strand Kit (SA Biosciences, MD). The cDNA samples were incubated with RT² SYBR Green/ROX qPCR Master Mix (SA Biosciences) and the expression of genes was determined using RT² Profiler for human ECM and adhesion molecules array on the 7900HT ABI 384-well block. Cycling conditions used were 10 min at 95 °C (1 cycle) and 15 s at 95 °C, 1 min at 60 °C (40 cycles). Gene expression was quantified following manufacturer’s instructions using the ΔΔCt method in which Ct refers to the fractional cycle number where the fluorescent signal reaches a detection threshold. The ΔCt was normalized using a total of five endogenous housekeeping genes (B2M, HPRT1, RPL13A, GADPH and ACTN). Fold change values were reported as 2^{-ΔΔCt} and when the value was less than one it was converted to its negative inverse to report downregulated genes. When Ct value was greater than 35, gene was considered nondetectable per manufacturer’s instructions.
5.3.9 Statistical Analysis

Data were analyzed using ANOVA. Means were generated and adjusted with Least Significant Difference using Statistical Analysis System software version 9.1. Significant differences were reported at $P$ values < 0.05.

5.4 Results

5.4.1 Lunasin caused cytotoxicity to different human CRC cells and their oxaliplatin resistant variants

Lunasin caused dose-dependent cytotoxicity to KM12L4, HT-29, HCT-116, RKO colon cancer cells and their OxR variants except for HT29OxR wherein no significant cytotoxic effect was observed when treated with up to 50 µM lunasin (Figures 5.1A and 5.1B). Among the parental cell lines used, lunasin most potently inhibited the growth of the metastatic KM12L4 colon cancer cells with an $IC_{50}$ of 13.0 µM. At a concentration as low as 1 µM, lunasin caused 19.8% inhibition of KM12L4 metastatic colon cancer growth ($P < 0.05$). At 50 µM, lunasin caused at least 90% reduction in the viability of KM12L4 cells. Also, lunasin caused a significant reduction in the viability of RKO ($IC_{50} = 21.6$ µM), HCT-116 ($IC_{50} = 26.3$ µM) and HT-29 ($IC_{50} = 61.7$ µM) CRC cells. Lunasin also caused a cytotoxic effect to KM12L4OxR ($IC_{50} = 34.7$ µM), RKOOxR ($IC_{50} = 38.9$ µM) and HCT116OxR ($IC_{50} = 31.6$ µM). On the other hand, lunasin up to 100 µM showed no cytotoxic effect to normal human colon fibroblast CCD-33Co (Figure 5.1A).

We demonstrated that the cytotoxic effect of lunasin correlated with the expression of $\alpha_5\beta_1$ integrin (Figure 5.1C) in different colon cancer cell lines. KM12L4 colon cancer cells highly expressed this integrin while HT-29 did not express $\alpha_5$ integrin. This is the first report on lunasin anti-cancer potential attributed to integrin signaling.
Lunasin synergistically interacted with oxaliplatin causing cytotoxicity in KM12L4 cells. (Figure 5.1D).

5.4.2 Lunasin caused G2/M cell cycle arrest on KM12L4 colon cancer cells

Lunasin, in a dose-dependent manner, increased the amount of cells at G2-phase of the cell cycle (Figure 5.2A) and increased the expression of cyclin dependent kinase inhibitors (CDKI) p21 and p27 (Figure 5.2B). Lunasin at 10 µM resulted in a 2.2-fold increase in the expression of p21 and 2.3-fold increase in the expression of p27. The same trends were obtained in the case of KM12L4OxR cells but at a higher concentration of lunasin (25 µM) (Figure 5.2C).

5.4.3 Lunasin promoted apoptosis on KM12L4 colon cancer cells

Figure 5.3A shows the percentage of KM12L4 colon cancer cells undergoing apoptosis in the presence or absence of lunasin after treatment for 24 h. Lunasin increased the amount of cells undergoing apoptosis from 13.6% (untreated) to 21.7%, 24.7% and 27.7% for cells treated with 5, 10 and 25 µM lunasin, respectively. Figure 3B shows representative pictures from cells treated with 10 and 25 µM lunasin for 24 h viewed using a fluorescence microscope. Untreated control cells showed normal morphology with intact cell structures while cells treated with lunasin showed characteristics of apoptosis such as nuclear condensation and DNA fragmentation. Also, the population of cells reduced significantly as seen in Figure 5.3B further confirming the cytotoxic effect of lunasin towards KM12L4 cells.

5.4.4 Lunasin activated the mitochondrial pathway of apoptosis

To further understand the mechanism involved in lunasin-mediated apoptosis, we measured the expression of proteins associated with the mitochondrial pathway of
apoptosis (Figures 5.4A and 5.4B). Treatment of KM12L4 with 10 µM lunasin resulted in the reduced expression of anti-apoptotic Bcl-2 by 2-fold while the same concentration of lunasin resulted in 1.5-fold decrease in KM12L4OxR. The expression of the pro-apoptotic Bax was increased by lunasin (10 µM) treatment by 2.2- and 2.3-fold in KM12L4 and KM12L4OxR cells, respectively. Lunasin (10 µM) increased the release of cytosolic cytochrome c, a major product of mitochondrial permeabilization as a result of Bax mitochondrial translocation, by 2.1-fold and 1.8-fold in KM12L4 and KM12L4OxR cells, respectively. In addition, the expression of the pro-apoptotic form of clusterin, nuclear clusterin, was also increased. The expression of the effector of apoptosis caspase-3 increased by 1.8- and 1.7-fold in KM12L4 and KM12L4OxR colon cancer cells, respectively, after treatment with lunasin (10 µM) (Figure 5.5A). As shown in Figure 5.5B, lunasin (5 and 10 µM) increased the activity of initiator caspases -2 and -9 but not caspase-8 indicating that lunasin activated the mitochondrial pathway of apoptosis but did not involve the activation of receptor-mediated apoptosis. Increase in the activity of caspase-9 resulted in an increased 2.3-fold activity of effector caspase-3 in KM12L4 cells treated with 10 µM lunasin when compared to untreated cells. We also elucidated whether lunasin can directly activate caspase-3 independent of caspase-9. When caspase-9 inhibitor was added into the cell culture of lunasin (10 µM) treatment, there was no upregulation of caspase-3 activity observed indicating that lunasin activation of caspase-3 was dependent of caspase-9 activation while caspase-9 was still active even in the presence of caspase-3 inhibitor.

5.4.5 Lunasin modified the expression of human extracellular matrix and adhesion genes
Table 1 summarizes the effect of lunasin on the expression of human ECM and adhesion genes in KM12L4 cells. Out of 62 genes that were detected by the array, lunasin caused upregulation of 48 genes and downregulation of 14 genes. The most downregulated genes included COL7A1 (10.10-fold), integrin β2 (8.19-fold), MMP10 (7.71-fold), SELE (6.53-fold) and integrin α5 (5.01-fold), while COL14A1 (11.62-fold) was the most upregulated gene. These results demonstrated the possible role of lunasin in mediating angiogenesis and metastasis of cancer cells by affecting genes associated with ECM and cell adhesion.

5.5 Discussion

Lunasin exhibited different cytotoxic effects against different CRC cell lines; being more effective against parental cell lines than against OxR forms. Lunasin most potently inhibited the growth of KM12L4 with an IC$_{50}$ of 13 µM. This is 5 times more potent than against HT-29 colon cancer wherein lunasin exhibited an IC$_{50}$ of 61.7 µM [10]. On the other hand, this IC$_{50}$ was close to a previous study wherein lunasin exhibited an IC$_{50}$ of 16 µM against L1210 leukemia cell lines [9].

We explored the possibility that the four colon cancer cells tested differentially expressed a certain type of integrin, a receptor for RGD as the recognizing sequence. We found that the cytotoxic effect of lunasin against colon cancer cells correlated with the expression of α5β1 integrin. Lunasin had the most potent effect on KM12L4 which highly expressed this integrin receptor; while HT-29 was the least susceptible to lunasin’s effect and did not express α5 integrin. Since lunasin features a unique RGD-motif known as a recognition sequence for integrins, we hypothesized that these colon cancer cells differentially expressed the α5β1 integrin. The α5β1 integrin is one of the most widely studied integrin in cancer research. It is important for cell adhesion [26] thereby
controlling cell migration, growth, proliferation and apoptosis [27]. In colon cancer, inhibition of the $\alpha_5\beta_1$ function resulted in reduction of CRC liver metastasis and improved survival in mice [28]. Also, targeting the $\alpha_5\beta_1$ integrin in colon cancer cells using a polyethylene glycolylated liposome RGD peptide PR_b resulted in its internalization via $\alpha_5\beta_1$-mediated mechanism and improved cytotoxic effect of 5-fluorouracil encapsulated stealth liposomes against CT26.WT CRC cells [29]. Moreover, transfection of the $\alpha_5$ integrin in HT-29 colon cancer cells resulted in suppression of apoptosis triggered by serum deprivation [30]. The expression of $\alpha_5\beta_1$ integrins also correlated with the invasive capability of different CRC cell lines and contributed to malignant progression in colon carcinoma [31]. Our results suggest that lunasin can be used as a potential adjuvant therapeutic agent against cancer cells highly expressing the $\alpha_5\beta_1$ integrin receptors thereby avoiding the side effects and resistant-development associated with the use of chemotherapeutic drugs alone.

Since lunasin most potently affected the proliferation of KM12L4 and KM12L4OxR, we determined its molecular basis. Lunasin affected cell cycle progression of the metastatic KM12L4 CRC cells, arresting the cell cycle at G2/M phase. This result is in accord with our previous studies in L1210 and HT-29 cancer cells [9, 10]. On the other hand, our results differ with other studies showing the capability of RGD-containing peptides to arrest cell cycle at G1-phase [32, 33] which can be attributed to the cyclic nature of the RGD-peptide used in these studies. Moreover, lunasin has a polyaspartic acid tail that caused mitotic arrest [1, 12]. The G2/M arrest caused by lunasin was accompanied by increased expressions of the CDKI p21 and p27. This observation was found in both parental and OxR variant forms of KM12L4 CRC cells, with higher
expression found in nonresistant cells. Previous studies showed that the loss of p21 and 
p27 was linked to drug resistance. Loss of p21 function was associated with a tamoxifen 
growth-inducing phenotype in breast cancer cells [34] while increase in p27 restored 
tamoxifen sensitivity in tamoxifen-resistant breast cancer cell lines [35]. Our results 
showed that lunasin can increase the expressions of p21 and p27 in KM12L4OxR which 
suggest that lunasin might sensitize back OxR colon cancer cells to oxaliplatin. 

Lunasin increased the amount of cells undergoing apoptosis and presented apoptotic-
morphological changes on KM12L4 CRC cells such as DNA fragmentation and nuclear 
condensation. This increase in apoptotic cells was accompanied by modification of 
expression of Bcl-2 family of proteins. Lunasin increased the expression of the pro-
apoptotic Bax in both KM12L4 and KM12L4OxR cells which might be attributed to 
blocked expression of Bcl-2 and increase expression of the pro-apoptotic nuclear 
clusterin (nCLU). This increase in the expression of nCLU might be attributed to 
concomitant increase in the expression of p21 as previous study in colon cancer cells 
showed that nCLU-mediated apoptosis was dependent on p21 [36]. Upon DNA damage, 
Ku70 is complexed with nCLU and goes to the nucleus leading to the release of Bax from 
Ku70-Bax complex and its translocation to the mitochondria [37]. The translocation of 
Bax into the mitochondria leads to increase permeabilization of mitochondrial membrane 
resulting in the release of death-promoting factor like cytochrome c. Lunasin treatment 
led to increase amount of released cytochrome c further establishing the series of 
apoptotic events that happened when KM12L4 and KM12L4OxR were treated with 
lunasin. This is followed by increase in the activity of caspase-9 leading to activation of 
the effector of apoptosis caspase-3 in KM12L4 cells with concomitant increased
expression of caspase-3. Several studies showed the capability of RGD containing peptides to induce apoptosis in a variety of cell lines. Recent investigations showed that RGDS inhibited the growth of melanoma cells with an adhesion-independent mechanism through internalization in melanoma cells and specific interaction with survivin [38] and induced caspase-8 and caspase-9 activity in human endothelial cells [39]. They suggested a mechanism wherein the RGD motif can recognize intracellular target via cell internalization leading to procaspase auto-processing and activation. This suggested mechanism is also supported by studies demonstrating the capability of RGD-containing peptides to promote apoptosis through direct caspase-3 activation [14, 40, 41]. Recent studies also showed the apoptosis-inducing effect of lunasin in breast cancer. Hsieh et al. [11] showed that lunasin inhibited cell proliferation and induced cell death in the breast tumor sections of a MDA-MB-231 xenograft breast cancer mouse model. The same group also reported that lunasin was able to sensitize human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induced apoptosis [42].

Lunasin modified the expression of human ECM and adhesion genes indicating the role of lunasin in angiogenesis and metastasis of cancer cells. Lunasin upregulated COL14A1 (11.62-fold), a molecule which when knock-down caused increased growth of renal cell carcinoma cell lines [43]. Also, COL14A1 methylation was associated with a poorer prognosis in renal cell carcinoma patients [43] and usually absent in the vicinity of invading tumors such as Kaposi sarcoma and oral squamous cell carcinoma [44]. On the other hand lunasin downregulated integrins α5 and β2, indicating that lunasin can actually participate in integrin signaling that can be attributed to its RGD motif. Previous studies showed that upregulation of α5 integrin is correlated with invasion and epithelial-
mesenchymal transition of CRC cells [45] and its inhibition resulted in decreased activation of the PI3K pathway and cell adhesion [46]. The expression of MMP10, a matrix metalloproteinase associated with metastasis [47] and tumor growth acceleration [48], was downregulated by lunasin by 7.71-fold. These results suggest that lunasin can actually participate in the modification of genes associated with angiogenesis and metastasis. It is therefore important to study further the molecular basis of the anti-invasive and anti-metastatic potential of lunasin in CRC.

In summary, this is the first report on the apoptosis-inducing property of lunasin in human metastatic colon cancer cells and its OxR variant suggesting its potential as an agent to combat metastatic colon cancer particularly in cases where resistance to chemotherapy develops.

5.6 References


6. V. P Dia, W. Wang, V. L. Oh, B. O. de Lumen, E. Gonzalez de Mejia, Isolation, purification and characterization of lunasin from defatted soybean flour and in


Table 5.1 Effect of lunasin on gene expression of extracellular matrix and cell adhesion in human KM12L4 colon cancer.

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**Extracellular Matrix Proteases**

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**Extracellular Matrix Protease Inhibitors**

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*Fold change was calculated as $2^{-\Delta\Delta C_{\text{t}}}$ and when the value was less than one it was converted to its negative inverse to report downregulated genes.*
Figure 5.1 Lunasin cytotoxic effect to different colon cancer cells correlated with the expression of $\alpha_5\beta_1$ integrin and promoted adhesion of KM12L4 colon cancer cells. (A) Parental colon cancer cells. (B) Oxaliplatin-resistant colon cancer cells. (C) Expression of $\alpha_5$ and $\beta_1$ integrins by different colon cancer cells. (D) Interaction study between lunasin and oxaliplatin in causing cytotoxicity to KM12L4 human colon cancer cells.
Figure 5.2 Lunasin arrested cell cycle progression at G2/M-phase of KM12L4 colon cancer cells and increased the expression of cyclin dependent kinase inhibitor p21 and p27 in KM12L4 and KM12L4OxR colon cancer cells. (A) Cell cycle progression analysis of cells as affected by lunasin treatment (B) p21 and p27 expression of KM12L4 colon cancer cells as affected by lunasin treatment. (C) p21 and p27 expressions of KM12L4OxR colon cancer cells as affected by lunasin treatment.
Figure 5.3 Lunasin induced apoptosis of KM12L4 colon cancer cells. (A) Apoptosis was measured after treatment of confluent cells for 24 h with different concentrations of lunasin. (B) Fluorescence microscopic images of KM12L4 colon cancer cells treated with lunasin at 0, 10 and 25 µM for 24 h. Cells treated with lunasin showed characteristics of apoptosis such as condensation (asterisks) and fragmentation (arrows) compared to untreated cells with normal nucleoli (triangle). All the images were taken at a scale of 20 µm.
Figure 5.4 Lunasin modified the expression of proteins associated with mitochondrial pathway of apoptosis in colon cancer cells. (A) KM12L4 and (B) KM12L4OxR.
Figure 5.5 Lunasin increases expression of caspase 3 in (A) KM12L4 and (B) KM12L4OxR and (C) induces caspase-9 and caspase-3 activity in KM12L4 colon cancer cells.
CHAPTER 6
LUNASIN SUPPRESSES FAK/ERK/NF-κB SIGNALING, BINDS α5β1 INTEGRIN
AND INHIBITS METASTASIS OF HUMAN COLON CANCER CELLS IN
VITRO AND IN VIVO

6.1 Abstract

The objective of this study was to determine the potential of lunasin purified from
defatted soybean flour to mediate the process of colon cancer metastasis using three
different human colon cancer cell lines in vitro and using a liver metastasis of colon
cancer (CRC) model in vivo. Lunasin internalized into the nucleus of KM12L4 colon
cancer cells after 72 h of treatment. Co-immunoprecipitation and Western blot showed
that lunasin binds with cellular α5β1 integrin. At 10 µM, lunasin inhibited the activation of
FAK by 28, 39 and 60% in RKO, HCT-116 and KM12L4 human colon cancer cells,
respectively. Also, it inhibited the activation of extracellular signal regulated kinase
[ERK] by 44% in HCT-116 cells and 61% in KM12L4 cells but not in RKO cells.
Inhibition of FAK autophosphorylation led to increased expression of the inhibitor of κB
by 1.8- 1.9- and 2.5 fold in RKO, HCT-116 and KM12L4 cells, respectively, and
decreased nuclear p50 NF-κB sub-unit expression by 70%. Inhibition of the constitutive
active NF-κB led to reduced migration of the colon cancer cells. An in vivo model of
CRC liver metastasis showed that lunasin administered at 4 mg/kg bw potentiated the
effect of the chemotherapeutic drug oxaliplatin in preventing the outgrowth of CRC
metastasis from spleen to the liver. Lunasin reduced the expression of proliferating cell
nuclear antigen (PCNA) and potentiated the effect of oxaliplatin. In conclusion, lunasin
inhibited metastasis of human colon cancer cells by suppressing FAK/ERK/NF-κB
signaling and by direct binding with $\alpha_5\beta_1$ integrin. Lunasin also potentiates the effect of oxaliplatin in preventing the outgrowth of CRC metastasis from spleen to the liver.

**6.2 Introduction**

Lunasin is a naturally occurring 43-amino acid peptide originally isolated from soybean. Its chemopreventive property is attributed to its capability to affect histone acetylation/deacetylation process [1], inhibit lipopolysaccharide (LPS) induced-inflammation [2-5] and activate apoptosis [6-9]. These bioactive properties are due to its unique amino acid sequence present on its structure. It features a polyaspartic acid tail with nine aspartic acid residues that directly bind to histones thereby affecting proper complex formation leading to mitotic arrest and cell death [1]. Moreover, it has a cell adhesion motif composed of arginine-glycine-aspartic acid [RGD] residues that causes its internalization into cells and attachment to extracellular matrix [ECM]. ECM controls different cellular processes including cell migration, proliferation and differentiation [10] and its protein components, mainly fibronectin, exerts its biological effect by binding cell surface receptors such as integrins. Integrins are heterodimeric glycoprotein receptors formed by a combination of different $\alpha$- and $\beta$-subunits and are ubiquitously expressed in human tissues with different combinations and ligand specificity [11]. Thus, integrins are involved in cellular processes like cell adhesion, migration, proliferation and angiogenesis. The $\alpha_5\beta_1$ integrin primarily binds to fibronectin to engage the cell and successively activate non-receptor tyrosine kinases, focal adhesion kinase [FAK] and Src thereby controlling tumorigenesis by promoting the proliferation and invasion of cancer cells [12]. Binding of integrins has emerged as a promising target for the development of compounds for cancer therapy [13]. In cancer, the most completely understood role of
α₅β₁ integrin is its ability to promote angiogenesis [14-17] thereby regulating the initial stage of cancer metastasis.

In 2008, over 1.2M new cases and 608,700 deaths was attributed to colorectal cancer [CRC] making it the third and second most common cancer in males and females, respectively [18]. It develops through a multi-stage progression of normal tissue into invasive cancer involving activation of oncogenes and inactivation of tumor-suppressor genes [19]. Through screening, surgery and classical chemotherapy, the incidence of CRC has been reduced and improved the survival rate of patients, however at least 90% of CRC deaths are caused by metastasis and not by primary solid tumors [20]. This is due, in part, to the fact that classic chemotherapy can only target the primary tumor mass and to the fact that the molecular events leading to tumor metastasis are only partially known. Moreover, continued use of chemotherapy is associated to adverse side effects and development of resistance, which may lead to patient fatality. It is therefore important to find non-toxic agents that can delay or prevent the process of cancer metastasis.

Our previous study showed the ability of lunasin to cause cytotoxicity and induce apoptosis in human colon cancer cells, including the highly metastatic KM12L4 cell line. Moreover, we found that lunasin can modify the expression of genes associated with ECM and cell adhesion [8]. In this study, we hypothesized that lunasin can inhibit the outgrowth of metastasis of human colon cancer cells via direct interaction with α₅β₁ integrin. Moreover, we hypothesized that lunasin can potentiate the anti-metastatic effect of oxaliplatin in vivo. We report here for the first time that lunasin can inhibit metastasis of human colon cancer cells by suppressing FAK/ERK/NF-κB signaling and binding with
α5β1 integrin. In addition, lunasin inhibits metastasis and potentiates the anti-metastatic effect of oxaliplatin in vivo.

6.3 Materials and methods

6.3.1 Materials

Human colon cancer cells HCT-116, KM12L4, RKO were obtained from Dr. Lee M. Ellis (MD Anderson Cancer Center, University of Texas). The metastatic KM12L4 cell line was established by injecting the parental cell line KM12C into the spleen of nude mice as previously reported [21]. Eagle's Minimum Essential Medium and 0.25% (w/v) Trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (Manassas, VA). Primary antibodies for actin (epitope mapping between amino acids 350-375), nucleolin (C23) (epitope mapping between amino acids 271-520), ERK (epitope mapping between amino acids 276-333), p-ERK (dual phosphorylation sites at Thr 202 and Tyr 204), FAK (epitope mapping between amino acids 903-1052), p-FAK (phosphorylation site at Tyr 397), IκB-α (epitope mapping for C-terminus), p50 (epitope mapping between amino acids 120-139), p65 (epitope mapping for N-terminus), and α5 integrin (epitope mapping for C-terminus), radio immunoprecipitation assay (RIPA) buffer and Protein A/G PLUS-Agarose Immunoprecipitation reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-mouse IgG and anti-rabbit IgG horseradish conjugate secondary antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Alexa-fluor 488, ITsignal FX, prolong gold antifade reagent and 4',6-diamidino-2-phenylindole [DAPI] stain were purchased from Invitrogen (Carlsbad, CA). Lunasin (at least 90% purity) was purified from defatted soybean flour as we have
previously reported [2, 3]. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

6.3.2 Cellular localization of lunasin by confocal immunofluorescence microscopy

Human KM12L4 colon cancer cells were seeded at a density of 1 x 10^3 cells per well in 8-well ibiDi plates and allowed to grow to 50-60% confluency for 72 h. Cells were treated with 1 µM lunasin for 24, 36, 48 and 72 h. Untreated cells served as 0 h treatment of lunasin. After corresponding time of treatment, cells were washed with fresh medium once, washed with PBS 3x and fixed with 4% paraformaldehyde (EMS, Hatfield, PA) for 30 min at room temperature. Cells were again washed with PBS 3x (5 min each) and permeabilized with 0.5% Triton X-100 for 15 min at room temperature, washed with PBS once and fixed with ultra-cold methanol for 15 min at -20 ºC. After fixing with methanol, cells were kept in PBS for 30 min at room temperature and blocked with ITsignal FX for 30 min. After blocking and a single wash with PBS, lunasin mouse monoclonal antibody (1:100 dilution) was added and incubated for 2 h at 37 ºC. After primary antibody incubation, cells were washed with PBS 3x (5 min each) at room temperature to remove unbound antibody. Anti-mouse secondary antibody conjugated with Alexa-fluor 488 (1:200 dilution) were then added and incubated in the dark for 90 min at 37 ºC. After washing with PBS 3x (5 min each), cells were stained with nucleic acid DAPI stain in the dark for 15 min at room temperature, washed with PBS 3x and mounted with antifade Prolong gold reagent and cured in the dark for 24 h at room temperature. After curing, cells were kept at 4 ºC until image analysis using a LSM 700 confocal microscope (Zeiss, Germany). Emission wavelengths used were 405 and 490 nm for DAPI and lunasin,
respectively. Imaged were taken at 3 independent fields per well for 3 independent replicates for cells treated with lunasin for 0, 24, 36, 48 and 72 h.

6.3.3 Co-immunoprecipitation of lunasin-α5β1 integrin complex interaction

KM12L4 cells were used to study the interaction between lunasin and α5β1 integrin as our previous study showed that KM12L4 highly expressed this type of integrin [8]. KM12L4 cells were seeded at 1 x 10^6 in 25 cm² flask and allowed to grow for 48 h at 37 °C in 5% CO₂/95% air. One flask was treated with 5 µM lunasin and one flask was treated with PBS as control for 24 h at 37 °C in 5% CO₂/95% air. After treatment, cells were washed with ice-cold PBS twice and 3 ml ice cold RIPA buffer were added and incubated for 10 min at 4 ºC. After RIPA buffer lysis, cells were disrupted by repeated aspiration using a 22-gauge needle and disrupted cells were transferred in 15 ml centrifuge tube. The flask was washed with 1-ml RIPA buffer and washing was combined with the original cell extract. Cellular debris was pelleted by centrifugation at 10,000 x g for 10 min at 4 ºC. Supernatant was transferred in a new 15-ml centrifuge tube and pre-cleared with normal IgG together and 20 µl of resuspended Protein A/G PlusAgarose beads for 30 min at 4 ºC. Afterwards, cells were pelleted at 1,000 x g for 5 min at 4 ºC and supernatant was collected. The protein content of the supernatant was determined using DC protein assay (Biorad, Hercules, CA). Approximately 500 µg total cell protein was placed in a 1.5 ml microcentrifuge tube and 2 µg of lunasin antibody and of α5 integrin antibody were added separately in different tubes. Cell lysate with normal IgG served as control. Incubation with primary antibody was allowed for 2 h at 4 ºC. After which, 20 µl of resuspended Protein A/G PlusAgarose beads were added, tubes were placed in an end-over-end rotator mixer and incubated overnight at 4 ºC. After incubation, immunoprecipitates were
collected by centrifugation at 1,000 x g for 5 min at 4 ºC. Supernatant was discarded and
the immunoprecipitate was washed with 1 ml RIPA buffer for 4 times with centrifugation
after every wash. Equal volume of immunoprecipitate and Laemlli sample buffer were
mixed and boiled for 2-3 min. Analysis of interaction was performed using
immunoblotting technique as described above for lunasin and α5β1 integrin. Analyses
were performed in three independent replicates.

6.3.4 Cell adhesion of KM12L4 colon cancer cells

Lunasin and bovine serum albumin [BSA] were dissolved in PBS and bound to a 96-well
plate for 3 h at 37 ºC in a CO2 incubator. The solution was then removed and blocked
with 0.1% albumin in PBS for another 30 min. The albumin was then removed and 1 x
10^4 KM12L4 colon cancer cells were added to each well. After 4 and 8 h incubation,
nonadherent cells were removed and adherent cells were washed 3 times with PBS. The
cells that adhered were measured using the MTS assay as previously reported [8].
Adhesion was reported as percentage relative to the amount of cells that adhered in wells
bound with BSA.

6.3.5 Western blot analysis of nuclear p50 and p65 NF-κB subunits

HCT-116, KM12L4 and RKO cells were seeded at a density of 2 x 10^5 cells per well in a
6-well plate for 24 h at 37 ºC in 5% CO2/95% air. After 24 h incubation, cells were
treated with 5 and 10 µM lunasin for 24 h. Cells treated with PBS served as control. After
treatment, cells were washed with PBS twice, trypsinized, washed again with PBS and
cytoplasmic and nuclear fractions were separated using NE-PER nuclear extraction kit
(Thermo Scientific, Pierce, IL) following manufacturer instructions. Equal volume of
nuclear fraction and Laemlli sample buffer with 5% β-mercaptoethanol were mixed and
boiled for 5 min. Equal amount of proteins was loaded in 4-20% Tris-HCl ready gels (Biorad Laboratories, Hercules, CA, USA) for protein separation. The separated proteins were transferred to PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min each) and incubated with nucleolin (C23), p50 and p65 primary antibodies (1:200) at 4 °C overnight. The membrane was washed again and incubated with anti-mouse IgG horseradish peroxidase conjugate secondary antibody for 2-3 h at room temperature. After incubation and repeated washings, the expression of nucleolin, p50 and p65 was visualized using chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK). Analyses were done for three independent replicates.

6.3.6 Western blot analysis of IκB-α, FAK, p-FAK, ERK and p-ERK

HCT-116, KM12L4 and RKO cells were seeded at a density of 2 x 10^5 cells per well in a 6-well plate for 24 h at 37 °C in 5% CO₂/95% air. After 24 h incubation, cells were treated with 5 and 10 µM lunasin for 24 h. Cells treated with PBS served as control. After treatment, cells were washed with PBS twice, trypsinized, suspended in lysis buffer composed of 62.5 mmol/l Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol and protease inhibitor cocktail (Thermo Scientific, Rockford, IL). The cell suspension was then sonicated and boiled for 5 min. Equal amount of proteins was loaded in 4-20% Tris-HCl ready gels (Biorad Laboratories, Hercules, CA, USA) for protein separation. The separated proteins were transferred to PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min each) and incubated with actin, IκB-α, ERK, p-ERK, FAK, p-FAK.
primary antibodies (1:200) at 4 °C overnight. The membrane was washed again and incubated with anti-mouse IgG horseradish peroxidase conjugate secondary antibody for actin, IκB-α, ERK, FAK, p-ERK and p-FAK for 2-3 h at room temperature. After incubation and repeated washings, the expression of actin, IκB-α, ERK, p-ERK, FAK, p-FAK was visualized using chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK). Analyses were done for three independent replicates.

6.3.7 Cell migration assay

To determine the effect of lunasin in cell migration, a scratch assay was performed as follows: RKO cells were used to determine the ability of lunasin to inhibit migration of cancer cells, as KM12L4 cells migrate poorly in in vitro assays as previously reported [22]. RKO cells were seeded at a density of 2 x 10^5 cells per well in a 6-well plate and allowed to grow to 70-80% confluency at 37 °C in 5% CO₂/95% air. After which, a scratch was made using a sterile 20 µl pipette tip. Cells were washed with PBS to remove any debris, the scratch was visualized using a phase contrast Apotome microscope and cells were treated with 10 µM lunasin for 24 h. Cells treated with PBS and doxycycline served as negative and positive controls, respectively. After 24 h treatment, the scratch was again visualized using phase contrast Apotome microscope. Migrated cells were quantified using Image analysis software (www.nih.gov). Analyses were done in three independent sites per well in three independent replicates.

6.3.8 In vivo model of colorectal cancer liver metastasis

The protocol was approved by the Institute of Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. Thirty seven male athymic mice 7 weeks old were purchased from Harlan Laboratories and acclimated for one week and 1,000,000
KM12L4 colon cancer cells suspended in 50 µL Hank's balanced salt solution were injected directly to the spleen of anesthetized mouse. After 4 days, mice were randomized into 4 groups as follows: 1) control administered with 100 µL PBS intraperitoneally as vehicle for 28 days after randomization (n = 10); 2) lunasin treated group was injected with lunasin intraperitoneally at 4 mg/kg bw daily suspended in 100 µL PBS for 28 days after randomization (n = 9); 3) oxaliplatin treated group was administered with oxaliplatin intraperitoneally at 5 mg/kg bw twice weekly suspended in 100 µL PBS for 28 days after randomization (n = 9); and 4) lunasin and oxaliplatin treated group administered with lunasin and oxaliplatin at the same dose and frequency as in groups 2 and 3 (n = 9). For the combined treatment group lunasin was administered intraperitoneally first and after approximately 1 h, oxaliplatin was given intraperitoneally. All groups received a standard AIN-93G powdered diet devoid of soy protein (Harlan Laboratories, Madison WI) along with the treatment. Mice were sacrificed by carbon dioxide asphyxia one day after 28 days of treatment. Total body weight and liver weight were determined during necropsy. The number of metastatic nodules on the liver was counted. Liver tumor was fixed in 10% formalin buffered solution for 24 h prior to tissue processing and paraffin embedding for immunohistochemical analyses.

**6.3.9 Hematoxylin and Eosin staining of paraffin embedded sections of liver**

Liver CRC tumors were stained with hematoxylin and eosin (H and E) by standard method. Stained slides were visualized using Nanozoomer Digital Pathology (Olympus Hamamatsu, Bridgewater, NJ).

**6.3.10 Immunohistochemical analyses**
Paraffin-embedded liver tumor tissue was cut at 5 µm thickness and transfer to a slide prior to immunohistochemical (IHC) analyses. IHC for PCNA and caspase-3 was performed following manufacturer’s protocol (Santa Cruz Biotechnology, CA). Stained slides were visualized using Nanozoomer Digital Pathology (Olympus Hamamatsu, Bridgewater, NJ). Intensity of staining was quantified using Teton machine (MediaCybernetics, Inc., Bethesda MD) equipped with Axio Vision analysis software (Carl Zeiss, Jena, Germany).

6.3.11 Statistical Analysis

For *in vitro* studies, data were analyzed using ANOVA. Means were generated and adjusted with Least Significant Difference using Statistical Analysis System software version 9.1. For the *in vivo* data, a non-parametric Mann-Whitney test was used to test for statistical differences between groups. Significant differences were reported at P values < 0.05.

6.4 Results

6.4.1 Lunasin internalized in human KM12L4 colon cancer cells

Figure 6.1 shows images from laser scanning microscope of human KM12L4 colon cancer cells treated with 1 µM of lunasin for 24, 36, 48 and 72 h. After 24 h of treatment, lunasin was localized to the cytoplasm of the cells. After 72 h of treatment, lunasin was also present in the nucleus of the cells.

6.4.2 Lunasin interacted with α5 integrin and adheres to KM12L4 human colon cancer cells

Co-immunoprecipitation experiment (Figure 6.2A) was used to determine the possible interaction between lunasin and α5 integrin receptor. We used α5 antibody to capture the
complex and looked for lunasin using immunoblotting technique. As shown in Figure 6.2B, normal IgG and PBS-treated cells did not respond with immunoblotting using lunasin antibody while lunasin-treated cells showed a positive response. Moreover, KM12L4 colon cancer cells adheres to lunasin lunasin relative to bovine serum albumin in a time- and dose-dependent manner up to 5 µM as shown in Figure 6.2C.

6.4.3 Lunasin inhibited FAK/ERK signaling on human colon cancer cells

As shown in Figures 6.3A and 6.3B, lunasin treatment resulted in the reduced expression of phosphorylated-FAK (p-FAK) with no concomitant effect on the expression of total FAK. This also resulted in the reduction of phosphorylated-ERK (p-ERK) in HCT-116 and KM12L4 colon cancer cells but not RKO cells (Figures 6.3A and 6.3C) indicating a possible different pathway by which lunasin affected NF-κB signaling in RKO colon cancer cells. The reduction in the expressions on p-FAK and p-ERK indicates the capability of lunasin to inhibit activation of this signaling pathway in colon cancer cells in vitro.

6.4.4 Lunasin inhibited constitutive NF-κB signaling on human colon cancer cells

Figure 6.4 shows the effect of lunasin treatment on the expression of IκB-α, a protein responsible for anchoring NF-κB into the cytoplasm thereby preventing its translocation into the nucleus. At 10 µM lunasin increased the expression of this protein by 1.8- 1.9- and 2.5 fold in RKO, HCT-116 and KM12L4 cells, respectively. This increase in the expression of IκB-α resulted in reduced expressions of p50 and p65 sub-units of NF-κB in the nucleus. Lunasin reduced the expression of p50 sub-unit more than p65 in all three colon cancer cells, with the most pronounced effect observed in KM12L4 colon cancer cells. The results of the co-immunoprecipitation experiment showed that lunasin interacts
with $\alpha_5\beta_1$ integrin which might explain the inhibitory effect of lunasin in FAK/ERK/NF-κB signaling.

### 6.4.5 Lunasin inhibited migration of human RKO colon cancer cells

Figure 6.5 shows the effect of lunasin on the migration of RKO cells. As shown, 10 $\mu$M lunasin after 24 h treatment inhibited migration of RKO cells by 33% relative to control. This inhibitory effect was lower than doxycycline at 5 $\mu$M, drug known to inhibit migration of cells, which inhibited migration of RKO cells by 54%.

### 6.4.6 Lunasin inhibited metastasis and potentiated the anti-metastatic effect of oxaliplatin in vivo

Lunasin administered at 4 mg/kg bw resulted in a significant inhibition of liver metastasis of colon cancer cells from 28 (PBS group) to 14 ($P = 0.047$) (Figure 6.6A). Also, lunasin potentiated the effect of oxaliplatin leading to an almost 6-fold reduction of liver metastasis when compared to PBS group ($P = 0.004$). This reduction in number of liver metastasis led to a decrease in tumor burden as measured by tumor weight over body weight (Figure 6.6B). As a measure of tumor mass, mean liver weight over body weight reduced significantly from 0.13 (PBS treatment) to 0.10 (lunasin treatment, $P = 0.039$), 0.08 (oxaliplatin treatment, $P = 0.011$) and 0.04 (L + O treatment, $P < 0.0001$).

Representatives of liver pictures from each group are presented in Figure 6.6C.

### 6.4.7 Hematoxylin and eosin staining and immunohistochemical analyses of liver-tumor tissue sections

Figure 6.7 shows representative slides of H and E staining of liver-tumor sections from each group of mice. Lunasin, oxaliplatin and their combination treatments resulted in an increase in the number of cells undergoing necrosis when compared to the group that
received PBS treatment. This can be explained by the reduction on the expression of PCNA in each treatment group when compared to the PBS group (Figure 6.8). Lunasin treatment resulted in 32% reduction of PCNA expression in the liver tumor tissue section (P = 0.046) while oxaliplatin treatment reduced PCNA expression by 60% (P = 0.001). L + O treatment most pronouncedly decrease PCNA expression by 86% (P = 0.001) when compared to the PBS group. This further supported the potentiating effect of lunasin when combined with oxaliplatin in killing the colon cancer tumor cells. On the other hand, lunasin, oxaliplatin and L + O treatment did not affect caspase-3 expression (Figure 6.9) on the liver-tumor tissue section when compared with PBS group.

6.5 Discussion

6.5.1 Lunasin inhibited metastasis in human colon cancer cells in vitro

Previous studies showed the chemopreventive property of lunasin against cancer by promoting apoptosis in different cancer cells including colon [7, 8], breast [23-25] and leukemia [6]. These biological activities can be attributed, partially, to the RGD cell adhesion motif present in the lunasin structure. The RGD motif is the structure responsible for its internalization into the cell as well as its interaction with ECM. This is the first report on the ability of lunasin to localize to the nucleus of human colon cancer cells. A previous study using synthetic lunasin showed that lunasin was internalized NIH 3T3 cells after 24 h of treatment [1], while lunasin purified from amaranth was found in the nucleus of the same cell line after 15 h [26].

Our results show that lunasin mostly localizes in the cytoplasm of KM12L4 cells after 24, 36 and 48 h of treatment and was found in the nucleus after 72 h. The difference in the internalization time might be attributed to the different cell type used in this study.
This internalization suggests that lunasin may function and affect molecules at an intracellular level and might explain the pro-apoptotic property of lunasin in cancer cells. Previous studies have shown the ability of lunasin to promote apoptosis by direct activation of caspase-3 [6] which can be a target of lunasin intracellularly. Also, a previous study of RGDS peptide showed that RGDS was able to internalize in melanoma cells in a time-dependent manner and interacted with several molecules that play a key role in the process of apoptosis, including survivin, procaspases-3, -8 and -9 [27]. The ability of lunasin to interact with ECM resulted in adhesion of human KM12L4 colon cancer cells to lunasin, indicating that lunasin interacted with KM12L4 cells. The RGD motif present in the lunasin structure is a recognition site for integrin receptors which can explain the increased adhesion of KM12L4 cells in the presence of lunasin, although this adhesion started to decrease at 10 µM lunasin, possibly because of its cytotoxic effect [8]. The adhesion of KM12L4 colon cancer cells to lunasin supports the idea of lunasin serving as an antagonist for ECM proteins for binding to the cell surface.

Endothelial cell proliferation and migration inside the tumor requires cell adhesion to macromolecules of the ECM, via cell membrane integrin receptors [28]. As a cell adhesion antagonist, lunasin might serve as an anti-angiogenic agent, as it can promote loss of cell anchorage by direct interaction with the ECM. The loss of cell anchorage can induce pro-apoptotic signals that lead to inhibition of angiogenesis [29] which supports the earlier reports on the pro-apoptotic property of lunasin. Moreover, if angiogenesis of tumor cells is inhibited, this can lead to suppression of outgrowth of metastasis of cancer cells.
Lunasin features an RGD motif, a recognition site for some integrin receptors. The α\textsubscript{5}β\textsubscript{1} integrin is a fibronectin receptor that can recognize the RGD motif on its ligand thereby leading to activation of integrin signaling, also involved in the cancer metastatic process. In order to determine whether the effect of lunasin on FAK/ERK/NF-κB signaling is associated with the RGD motif of the lunasin structure, we hypothesized that lunasin inhibits integrin signaling by direct binding with α\textsubscript{5}β\textsubscript{1} integrin. The co-immunoprecipitation results showed the ability of lunasin to interact with α\textsubscript{5}β\textsubscript{1} integrin, further supporting our hypothesis that lunasin can interact with the ECM via integrin receptors. It has been previously reported that soluble RGD-containing peptides can alter integrin-ECM interactions by binding and consequently blocking the particular integrin [30, 31]. Our results suggest that lunasin might be able to affect signaling downstream of integrin by blocking interaction of α\textsubscript{5}β\textsubscript{1} to ECM proteins. To further elucidate the mechanism by which lunasin affects integrin-mediated signaling, we evaluated the effect of lunasin on the expression of proteins associated with integrin-downstream signaling. Upon anchorage of integrins, the cytoplasmic FAK residing at adhesion sites plays a principal role in signaling. FAK gets autophosphorylated leading to activation of multiple pathways including ERK [32]. Our present results showed that lunasin downregulates the phosphorylation of FAK in three different human colon cancer cell lines without affecting the level of total FAK, further supporting the effect of lunasin on integrin signaling. Several studies have shown the ability of soluble RGD peptide as well as ECM proteins with RGD motif to affect FAK phosphorylation. Sohn et al. [33] showed that recombinant saxatilin (20 µM), a disintegrin from snake venom containing the RGD motif, was able to reduce the phosphorylation of FAK after 3 h of treatment with
dramatic decrease after 24 h of treatment. This inhibition was attributed to the ability of saxatilin to disassemble focal adhesion via paxillin degradation. Moreover, addition of soluble peptide led to activation of apoptosis and reduced autophosphorylation of FAK [34]. Another RGD-containing disintegrin, echistatin (20 µg/ml) reduced phosphorylation of FAK after 3 h of treatment [35]. Since FAK phosphorylation can lead to activation of multiple pathways including ERK signaling, we determined the effect of lunasin on the level of phosphorylated ERK in colon cancer cells. ERK activation can lead to activation of inhibitor of kappa B (IκB) kinase, an enzyme that phosphorylates IκB.

Phosphorylation of IκB targets its ubiquitination and degradation by the proteasome resulting in the activation of NF-κB and subsequent localization to the nucleus and transcription. Our results showed that constitutive NF-κB activation in colon cancer cells can be suppressed by lunasin treatment. Analysis on the expression of IκB-α showed that lunasin increased the expression of this protein thereby inhibiting the activation and subsequent nuclear translocation of p50 and p65 sub-units of NF-κB. This is then supported by our Western blot results showing that nuclear expressions of p50 and p65 sub-units were reduced upon lunasin treatment in the three colon cancer cell lines.

Constitutive NF-κB signaling has been reported previously in different colon cancer cell lines. Yang et al. [36] found that NF-κB is expressed in the nucleus of KM12L4 cells and its expression was not affected by development of resistance to oxaliplatin. This constitutively active NF-κB was also previously found in RKO [36] and HCT-116 [37, 38] colon cancer cells. Our results show that this lunasin-suppressed activity of NF-κB resulted in the prevention of migration of colon cancer cells (Figure 6.5). Previous studies have shown the ability of soluble RGD peptide to affect ERK/NF-κB signaling in
a variety of cell lines. Tan et al. [39] showed that cyclic RGD as low as 30 nM was able to inhibit connective tissue growth factor-associated migration of human chondrosarcoma cells. Moreover, they showed that this migration inhibitory effect of RGD peptide is through inhibition of ERK phosphorylation and NF-κB activation leading to reduced expression of matrix metalloproteinase (MMP)-13. Another study showed that fibulin 5, an ECM protein with conserved RGD motif, was able to inhibit serum-stimulated ERK and FAK phosphorylation via an RGD-dependent mechanism. This resulted in reduced lung cancer cell invasion though inhibition of MMP-7 [40]. The same results were found by Kudirka et al. [41] and Berken et al. [42] reporting that RGD peptide was able to inhibit extracellular nucleotide-induced and asbestos-induced ERK phosphorylation, respectively. In colon cancer, RGD peptides also demonstrated various effects on the metastatic process. A linear RGD peptide at a concentration ranging from 0.2 to 0.5 µM was able to inhibit adhesive interactions between circulating tumor cells and hepatic sinusoids in vivo [43]. Haier et al. [44] showed that cyclic RGD was able to inhibit tumor growth and reduce tumor load in chemically-induced colon carcinomas. Moreover, a cyclic RGD peptide was able to reduce intratumoral microvessel density in human colon cancer cell-inoculated mice [45]. Our result showed that 5 µM lunasin, extracted from natural sources was needed to observe these biological effects. This concentration difference can be explained, in part, by the differences in the structure and forms of the RGD peptides used in previous studies. These previous studies as well as our new results provide further evidence on the potential of RGD-containing peptides to mediate and inhibit the outgrowth of metastasis of cancer cells.
6.5.2 Lunasin inhibited and potentiated the effect of oxaliplatin in *in vivo* model of CRC liver metastasis

Metastasis, the spread of primary tumors to distant organs, is considered as the most life-limiting aspect of most types of cancer. It is a very inefficient process consisting of acquisition of motility and invasiveness of cancer cells, detachment and migration from the primary tumor, blood and lymphatic invasion, distant vascular endothelial system attachment, extravasation and growth and proliferation in the distant site [46, 47]. The liver is a common target organ for the metastasis of gastrointestinal cancers because tumor cells are disseminated into the circulation where they are transported by the blood to the liver [48-51]. Moreover, the sinusoidal endothelial layer is characterized by an incomplete covering making the underlying ECM components directly accessible to circulating cells [52]. In metastatic CRC, the 5-year survival rate is only approximately 8% [53]. CRC mortality is strongly associated with patients that developed liver metastasis. As such looking for agents that can prevent the outgrowth of colon cancer cell metastasis to the liver is important to effectively manage the disease. The spleen implantation model of CRC liver metastasis is a widely used model to study the effects of different markers and chemotherapy on CRC metastasis. We evaluated the effect of lunasin on this experimental model of CRC metastasis mainly on the capability of KM12L4 colon cancer cells to attach or extravasate in the mouse liver. Our findings showed that lunasin was able to reduce the tumor burden and number of liver metastatic nodules when compared to PBS treatment. Moreover, lunasin in combination with oxaliplatin was better in reducing tumor burden and liver metastasis when compared to either lunasin or oxaliplatin alone. This suggests that lunasin can enhance the effect of
oxaliplatin in preventing the outgrowth of colon cancer metastasis. IHC staining of liver-tumor tissue sections showed that lunasin, oxaliplatin and L + O treatments resulted in the reduction of PCNA expression but did not affect caspase-3 expression. This indicates that lunasin mode of action in reducing the tumor burden in mouse liver is through inhibition of cell proliferation leading to cancer cells necrosis rather than apoptosis.

The mechanism on how lunasin potentiates the effect of oxaliplatin or how it sensitizes cancer cells to the cytotoxic effect of oxaliplatin is not yet studied. It can be speculated that this potentiation or sensitization effect of lunasin is attributed to the ability of lunasin to modify tumor microenvironment especially the extracellular matrix by virtue of its RGD motif. Our in vitro data showed that KM12L4 colon cancer cells expressed the $\alpha_5\beta_1$ integrin, a receptor with recognition site for RGD sequence. Moreover, our co-IP experiment showed that lunasin physically interacted to this integrin receptor. The liver has an attenuated ECM which is mainly composed of fibronectin [54], a natural ligand for $\alpha_5\beta_1$ integrin. We proposed here that lunasin, through its RGD motif, blocked the $\alpha_5\beta_1$ integrin receptors expressed on the surface of KM12L4 colon cancer cells thereby preventing its attachment and subsequent extravasation of the liver tissue via its natural ligand fibronectin. When these cancer cells are not able to attach to a specific site, this makes their surroundings unsuitable for their growth (can be due to oxygen deprivation or lack of ability to produce growth factors and promote angiogenesis) which can cause cell arrest and eventually cell death. Moreover, in the presence of circulating cytotoxic agents such as oxaliplatin, these unattached cancer cells can be killed easily. These hypotheses are actually supported by our in vivo data showing
that lunasin itself can cause reduction of tumor burden in the liver and combining it with oxaliplatin resulted in a more pronounced reduction in CRC liver metastasis.

In conclusion, the results suggest that lunasin, a peptide with a RGD motif, can be used as a potential integrin antagonist thereby preventing the attachment and extravasation of colon cancer cells leading to its anti-metastatic effect. Moreover, lunasin can be used as an adjuvant in patients with metastatic CRC undergoing chemotherapy treatment. The \textit{in vitro} and \textit{in vivo} data presented in this research suggests a potential use of lunasin in prolonging survival of patients with metastatic CRC.

\textbf{6.6 References}


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Figure 6.1 Lunasin internalizes into KM12L4 colon cancer cells. KM12L4 colon cancer cells were treated with 1 µM lunasin for 24, 36, 48 and 72 h. After treatment, cells were fixed and treated with antibodies for lunasin for confocal immunofluorescence microscopy. Lunasin internalizes into the cells after 24 h and localizes into the cytoplasm as marked by arrowheads and translocates into the nucleus of the cells (n = 3) as marked by arrows showing high intensity of green fluorescence in the KM12L4 nucleus. (A) DAPI-stained cells for nucleus (B) Lunasin mouse monoclonal antibody and Alexa-Fluor 488-conjugated secondary antibody-stained cells (C) Composite of images in A and B. Image analysis were performed using LSM 700 confocal microscope (Zeiss, Germany); emission wavelengths used were 405 and 490 nm for DAPI and lunasin, respectively. Imaged were taken at three independent fields per well for 3 independent replicates for cells treated with lunasin for 0, 24, 36, 48 and 72 h.
Figure 6.2 Lunasin directly binds with α5 integrin and promoted adhesion of KM12L4 colon cancer cells. (A) Schematic diagram use for co-immunoprecipitation experiment. Lunasin physically interacts with α5 integrin. KM12L4 colon cancer cells were treated with 5 µM lunasin for 24 h, harvested by RIPA lysis buffer and lunasin-α5 complex was co-immunoprecipitated using Protein A/G Plus Agarose beads using α5 (B) antibody (n = 3). (C) Lunasin promoted cell adhesion of KM12L4 colon cancer cells (n = 3, P < 0.05).
Figure 6.3 Lunasin inhibits FAK/ERK signaling in colon cancer cells. HCT-116, KM12L4 and RKO colon cancer cells were treated with lunasin for 24 h and p-FAK, FAK, p-ERK1/2 and ERK1/2 were determined by Western blot. A) Representative blots of p-FAK, FAK, p-ERK1/2 and ERK 2 from three independent experiments. In a cell line, means of p-FAK expression (B) and p-ERK1/2 expression (C) with different letters are significantly different from each other (n = 3, P < 0.05).
Figure 6.4 Lunasin inhibits constitutive NF-κB signaling in colon cancer cells. HCT-116, KM12L4 and RKO colon cancer cells were treated with 5 and 10 µM lunasin for 24 h and expression of IκB-α, nuclear p50 and p65 NF-κ sub-units were determined by Western blot. (A) Representative blots of IκB-α, actin, p50, p65 and nucleolin from three independent experiments. In a cell line, means of IκB-α (B), p50 (C) and p65 (D) with different letters are significantly different from each other (n = 3, P < 0.05).
Figure 6.5 Lunasin inhibits the migration of RKO colon cancer cells. A scratch was made in RKO cells and treated with 10 μM lunasin for 24 h. Images were taken using phase-contrast Apotome microscope immediately after the scratch was made (A) and after 24 h treatment (B). Doxycycline (5 μM) was used as a positive control (n = 3, P < 0.05). Number in parenthesis indicates the integrated density of cells (IDC) in the scratch as measured by ImageJ software. (C) Relative migration of cells treated with lunasin (10 μM) and doxycycline (5 μM) compared to PBS-treated (control) cells.
Figure 6.6 Lunasin inhibits metastasis and potentiates the anti-metastatic effect of oxaliplatin in spleen-implantation model of metastatic colorectal cancer in vivo.

KM12L4 cells ($1 \times 10^6$) were inoculated into the spleen of male nude mice and after 4 days mice were randomized into 4 groups. One group received PBS, lunasin (4 mg/kg bw, everyday) oxaliplatin (5 mg/kg bw twice a week) and combination (L + O, same dose and frequency as previous) for 28 days. At day 29, mice were killed and number of liver metastasis was counted. Lunasin reduced the number of metastasis and potentiates the effect of oxaliplatin (A). Asterisks mean statistically significant differences for lunasin ($P = 0.047$), oxaliplatin ($P = 0.039$) and L + O ($P = 0.004$) vs PBS (as determined by Mann-Whitney analysis). Lunasin reduced tumor mass in the liver and potentiates the effect of oxaliplatin. (B). Asterisks mean statistically significant differences for lunasin ($P = 0.039$), oxaliplatin ($P = 0.011$) and L + O ($P < 0.0001$) vs PBS (as determined by Mann-Whitney analysis). Representative pictures of liver are presented (C) from the four groups.
Figure 6.7 Lunasin modifies liver-tumor tissue derived from KM12L4 cells morphology. Hematoxylin and eosin staining of liver-tumor tissue indicated that lunasin, oxaliplatin and L + O increased the number of cells undergoing necrosis when compared to PBS. Cells undergoing necrosis are circled and marked with arrowhead in the figure.
Figure 6.8 Lunasin reduces the expression of proliferating cell nuclear antigen in liver-tumor tissue derived from KM12L4 cells. Immunohistochemical staining for PCNA showed that lunasin and oxaliplatin treatment resulted in the reduction of PCNA expression when compared to PBS. Moreover, L + O treatment showed a remarked reduction of PCNA expression. Asterisks indicate statistically significant differences in PCNA expression for lunasin (P = 0.046), oxaliplatin (P = 0.001) and L + O (P = 0.001) vs PBS (as determined by Mann-Whitney analysis).
Figure 6.9 Lunasin, oxaliplatin and their combination do not affect the expression of cleaved caspase-3 in liver-tumor tissue derived from KM12L4 cells as measured by immunohistochemical staining. Lunasin, oxaliplatin and L + O treatment are not statistically different vs PBS (P > 0.05 as determined by Mann-Whitney analysis).
CHAPTER 7

CONCLUSIONS

- Lunasin can be isolated and purified (> 90%) from defatted soybean flour using a combination of ion-exchange chromatography, size-exclusion chromatography and ultrafiltration techniques.

- Lunasin isolated from defatted soybean flour caused cytotoxicity to different human colon cancer cells and its potency correlated with the expression of \( \alpha_5 \) integrin \textit{in vitro}.

- Lunasin induced G2/M cell cycle arrest in human colon cancer cells by increasing expression of the cyclin dependent kinase inhibitor p21 \textit{in vitro}.

- Lunasin caused apoptosis of human colon cancer cells by activating the mitochondrial pathway of apoptosis \textit{in vitro}.

- Lunasin prevented the outgrowth of metastasis by suppressing FAK/ERK/NF-\( \kappa \)B signaling and binding of \( \alpha_5 \) integrin \textit{in vitro}.

- Lunasin inhibited metastasis by reducing expression of proliferating cell nuclear antigen \textit{in vivo}.

- Lunasin potentiated the effect of the chemotherapeutic drug oxaliplatin in preventing the outgrowth of colon cancer cell metastasis in CRC liver metastasis model \textit{in vivo}. 
CHAPTER 8
INTEGRATION AND FUTURE WORK

The overall objective of this research was to evaluate the anticolon cancer potential of lunasin isolated from defatted soybean flour. Lunasin (>90% purity) was isolated from defatted soybean flour by a combination of chromatographic and ultrafiltration techniques. Soybean lunasin caused a cytotoxic effect to human colon cancer cells through cell cycle arrest and induction of the mitochondrial pathway of apoptosis. This can be attributed to the ability of lunasin to internalize into the cell and interact with α5β1 integrin. Moreover, lunasin caused inhibition of the FAK/ERK/NF-κB signaling in CRC cells leading to prevention of CRC metastasis. In vivo, lunasin prevented CRC liver metastasis and potentiated the anti-metastatic effect of oxaliplatin, suggesting that lunasin can be used as an adjuvant for patients suffering from metastatic CRC.

Cancer cell activate survival signaling pathways in order to proliferate and metastasize in distant organs. In the absence of any external factors, cancer cells escape programmed cell death by several routes. We propose that in the absence of lunasin CRC cells survive through escaping of mitochondrial pathway-mediated cell death and through activation of the FAK/ERK/NF-κB signaling. Upon activation of FAK/ERK signaling, ERK blocked the expression of the p21 protein leading to cell survival primarily due to nonmaturation of the precursor of nuclear clusterin (pnCLU). In the absence of mature nuclear clusterin (nCLU), Bax is sequestered by the pro-survival secretory clusterin (sCLU) and Ku70, Bax cannot be translocated to the mitochondria thereby preventing the intrinsic pathway-mediated cell death resulting in cell survival. Moreover, the high
expression of the anti-apoptotic Bcl-2 in CRC cells also leads to cell survival. In the presence of lunasin, the expression of the cyclin dependent kinase inhibitor p21 is induced which can be attributed to blocked ERK signaling leading to expression of the nuclear clusterin (nCLU) isoform. nCLU then breaks the complex sCLU-Ku70-Bax which causes translocation of Bax into the mitochondria leading to mitochondrial permeabilization. Upon permeabilization, cytochrome c is released into the cytosol and forms a complex with the apoptosis activating factor-1 (Apaf-1) and caspase-9, leading to cleavage and activation of caspase-9. Caspase-9 activation results in cleavage of caspase-3 which then activates the mitochondrial pathway-mediated cell death. Integrin engagement of the extracellular matrix protein such as fibronectin can also lead to a pro-survival signal. Fibronectin can actually engage the α\textsubscript{5}β\textsubscript{1} integrin which leads to the phosphorylation of the focal adhesion kinase (FAK) and extracellular regulated kinase (ERK). Activation of the FAK/ERK signaling pathway can cause translocation of NF-κB sub-units to the nucleus thereby promoting transcription of proteins associated with cell survival and metastasis. In the presence of lunasin, the engagement of the α\textsubscript{5}β\textsubscript{1} integrin by fibronectin is blocked which results in the reduced autophosphorylation of FAK. Reduction of phosphorylated FAK expression leads to reduce activation of ERK, resulting in reduced nuclear translocation of NF-κB. When NF-κB is kept in the cytosol, transcription of genes associated with cell survival and metastasis is reduced leading to reduced cell migration. The proposed mechanism by which lunasin induces apoptosis and prevents the outgrowth of metastasis of colon cancer cells is presented in Figure 8.1.

This research presented several novel biological properties of lunasin, a naturally occurring chemopreventive peptide found in soybean and other plant sources. For the
first time, it showed that lunasin has the potential to modulate CRC and possibly to be used as an adjuvant therapy in metastatic CRC. An *in vitro* model of CRC showed that lunasin induced the mitochondrial pathway of apoptosis which can be attributed to its ability to internalize into CRC cell. Moreover, lunasin prevented metastasis through inhibition of FAK/ERK/NF-κB signaling and by direct binding with α5β1 integrin. An *in vivo* model of CRC liver metastasis showed that daily intraperitoneal administration of lunasin at 4 mg/kg body weight for 28 days prevented the outgrowth of KM12L4 cells to form liver metastasis by reducing the expression of proliferating cell nuclear antigen. We chose a dose equivalent to the amount of lunasin present in 25 g of

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**Figure 8.1 Proposed mechanism by which lunasin induced apoptosis and prevented outgrowth of colon cancer metastasis.**
soy protein for an average person weighing 150 lbs. We administered approximately 100 µg of lunasin intraperitoneally which went directly in the circulation. Taking into consideration the mouse blood volume of approximately 2 mL, the greatest concentration of lunasin in the circulation was approximately 10 µM. This circulating lunasin concentration is very close to lunasin’s IC$_{50}$ of 13 µM for causing cytotoxicity to KM12L4 colon cancer cells in vitro. These values showed a correlation between in vitro and in vivo studies. Moreover, lunasin potentiated the anti-metastatic effect of oxaliplatin, a chemotherapeutic drug typically administered to patients with metastatic CRC. The results of this research highlight the potential for lunasin to mediate the process of colorectal carcinogenesis by inducing apoptosis and preventing the outgrowth of metastasis.

It is suggested that in the future further studies of the chemopreventive effect of lunasin in CRC are warranted. It is interesting to see if daily consumption of lunasin can prevent and delay the onset of CRC in chemically- and inflammatory-induced CRC. Moreover, the combination of a higher concentration of lunasin, probably given at 8 mg/kg body weight, and a lower concentration of oxaliplatin at half the actual dose currently given to patients with metastatic CRC warrants further study. It would be interesting to see if lunasin at a higher dose can sensitize colon cancer cells to a lower dose of oxaliplatin thereby avoiding cytotoxic side effects associated with the administration of the latter. The use of higher concentration of lunasin in the form of concentrated lunasin in the diet is needed to see the effect of digestion and absorption on the biological activity of lunasin. In addition, the results of this research can be used as a
basis for future clinical trials and for the potential use of lunasin as a dietary bioactive food component to reduce colon cancer risk in the general population.
APPENDIX

EXPERIMENTAL PROCEDURES

1. Bio-Rad DC protein assay

   **Reference:** Bio-Rad Protein Assay Instruction Manual

   **Materials:**
   - Reagent A and reagent B from Bio-Rad
   - Bovine serum albumin (BSA)
   - Tris-buffered saline (TBS)

   **Procedure:**
   a. Dissolve one packet of 0.05 M TBS, pH 8.0 in 1-L of deionized water (ddH$_2$O).
   b. Prepare BSA standard curve using TBS solution as follows: Weigh 75 mg of BSA and dissolve in 50-mL TBS solution. This is your stock solution with a concentration of 1500 µg/mL. Prepare the other solutions using a serial dilution as follow:
      
      | Concentration (µg/mL) | 1500 | 1200 | 900 | 600 | 300 | 100 | 30 | 10 |
      |------------------------|------|------|-----|-----|-----|-----|----|----|
      | µL                     | 120  | 90   | 60  | 30  | 20  | 10  | 3  | 1  |

   c. Pipet 5 µL standards, TBS and samples (dissolved in TBS) into a clean, dry 96-well plate in triplicate.
   d. Add 25 µL of reagent A into each well.
   e. Add 200 µL of reagent B into each well. Agitate gently and incubate for 15 min at room temperature.
   f. Read absorbance at 690 nm using the ELX 800 Biotek microplate reader. The absorbance is stable for about an hour.
g. Construct a standard curve with BSA standards.
h. Calculate protein concentration of each sample using the BSA standard curve.

2. **Enzyme-linked immunosorbent assay (ELISA) for lunasin concentration determination**

**Reference:**

**Materials:**
Lunasin standard: 8 to 120 ng/mL (prepare in TBS)
Washing solution: Dissolve 1 packet of 0.01 M PBS, pH 7.4, with 0.05% Tween-20 in 1-L of ddH₂O.
1% Tween 20-TBS (1% TBST): Dissolve one packet of 0.05 M TBS, pH 8.0 in 1-L of ddH₂O and add 10-mL of Tween 20.
Blocking solution (5% BSA, 30-mL per plate): Weigh 1.5 mg BSA and dissolve in 30-mL 1% TBST.
Antibody solution (3% BSA, 10-mL per plate): Weigh 0.3g BSA and dissolve in 10-mL 1% TBST.
19 N NaOH: Dissolve 76 g of NaOH pellet in 100-mL ddH₂O.
PNPP buffer (0.1 M glycine, 1 mM MaCl₂, 1 mM ZnCl₂, pH 10.4): Weigh and dissolve the following in 980-mL ddH₂O: 7.51 g glycine, 203 mg MgCl₂ and 136 ZnCl₂. Adjust the pH to 10.4 using 19 N NaOH and adjust the volume to 1-L with ddH₂O.
Primary antibody: Mix 1-µL of lunasin mouse monoclonal antibody for every 4-mL of antibody solution (1:4000 dilution).

Secondary antibody: Mix 1-µL of alkaline phosphatase conjugate antimouse IgG for every 7-mL of antibody solution (1:7000).

PNPP solution: Dissolve 1 tablet (20 mg) of PNPP in 20-mL PNPP buffer.

**Procedure:**

a. Make proper dilution of the samples in TBS.

b. Measure 100 µL of the standard, TBS and sample into Nunc Maxisorp 96-well plate.

c. Incubate plate at 4 °C overnight, approximately 14 h.

d. Wash the plate using Bio-Tek plate washer program 18.

e. Block each well by adding 250-300 µL of 5% BSA, incubate for 1 hr at room temperature.

f. Wash as in step d.

g. Add 50 µL of the primary antibody solution to each well and incubate for 1 hr at room temperature.

h. Wash as in step d.

i. Add 50 µL of the secondary antibody solution to each well and incubate for 1 hr at room temperature.

j. Wash as in step d.

k. Add 100 µL of PNPP solution to each well, incubate for 20 min at room temperature and read the absorbance at 405 nm using Bio-Tek microplate
reader. After 5 min, add 100 µL of 3 N NaOH, incubate for another 10 min at room temperature and read the absorbance at 405 nm.

l. Construct the standard curve using the lunasin standard solutions.

m. Calculate lunasin concentration of each sample taking into account all dilutions made.

3. **Western blot protocol using the Pharmacia LKB-Phast system for lunasin**

**Materials:**

Sample loading buffer: Add 20-µL β-mercaptoethanol to 980 µL Tricine sample buffer

Towbin’s transfer buffer: Weigh 3.03 g Tris and 14.4 g glycine and dissolve in 200-mL methanol. Adjust volume to 1-L with ddH₂O.

Washing solution (0.1% Tween 20-TBS, 0.1% TBST). Dissolve 1 packet of TBS in 1L ddH₂O and add 1-mL Tween 20.

Blocking solution: Dissolve 0.6 g of ECL Advance Blocking Agent in 30-mL of washing solution (good for one gel/membrane).

Primary antibody: Mix 2 µL of lunasin mouse monoclonal antibody for every 10-mL of blocking solution.

Secondary antibody: Mix 1 µL anti-mouse horseradish (HRP) conjugate for every 10-mL of blocking solution.

GE Healthcare ECL chemiluminescence reagent

**Procedure:**
a. Sample Preparation: Dilute sample with 1:2 sample loading buffer, mix and boil for 5 min. Vortex and centrifuge briefly using the table top mini centrifuge.

b. Electrophoresis: Apply sample on 8-25% Phast gel using 8/1 or 6/4 sample applicator. Run gel at 250 V, 10 mA, 3.0 W 0065 Vh at 15 °C.

c. Blotting: Carefully remove the gel from the Phast system and cut the hard plastic using the gel cutter. Soak the gel in Towbin’s transfer buffer. Soak 2 pieces of filter paper in transfer buffer. Replace the electrophoretic assembly in the Phast system with the blotting accessory. Place one filter paper on the black surface of the blotting accessory. Place on top of the filter paper a piece of PVDF membrane (previously wet in methanol and soak in transfer buffer for at least 5 min). Put gel facing down onto the membrane (hard plastic on top). Remove the hard plastic and place the other filter paper on top of the gel. Place weighted cathode on top of the filter paper. Run at 20 V, 25.0 mA, 1.0 W, 0005h at 15 °C.

d. Antibody incubations: Block the membrane in the blocking solution for 1 hr. Wash the membrane with washing solution for at least 3 times, 5 min each. Incubate with primary antibody overnight at 4 °C on a shaker. Wash 3 times, 5 min each with washing solution. Incubate with secondary antibody for 1 hr at room temperature. Wash 3 times, 5 min each with washing solution. Leave the membrane in the washing solution until ready for detection.
e. Detection: Prepare chemiluminescent reagent by mixing 0.5 mL of solution A and 0.5 mL solution B. Apply the reagent on the membrane. Read with Kodak Image Station 440 CF.

4. Thawing protocol for human colon cancer cells
   a. Prepare complete MEM medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% vitamin mix, and 1% glutamine. Measure 50-mL in a sterile media bottle and pre-heat at 37 °C.
   b. Take 1 vial of human colon cancer cells from the liquid nitrogen tank.
   c. Thaw the vial at 37 °C for approximately 2 min.
   d. Transfer the content of the vial in 15-mL sterile centrifuge tube and add 4-mL of the warmed media.
   e. Centrifuge at 125 x g for 7 min.
   f. Take out the supernatant and add 10-mL of the warmed media. Resuspend the cell pellet in the media.
   g. Transfer the content of the 15-mL sterile centrifuge tube in T-25 flask.
   h. Incubate at 37 °C, 5% CO₂/95% air.
   i. The cells are ready for subculture in 2-3 days.

5. Subculture protocol for human colon cancer cells
   a. Warm complete media at 37 °C.
   b. Take the flask out of the incubator.
c. Aspirate the growth media from the flask. Add 2-3 mL trypsin solution and aspirate. Add another 5-6 mL trypsin solution and aspirate. Put back the flask in the incubator for 3-5 min.

d. Take the flask out of the incubator, check the cells under the microscope if they are rounded and moving/detached from the surface of the flask.

e. Add 5-mL of the warmed media (more if the cells are too dense). Re-suspend the cells using a Pasteur pipette.

f. Measure 50 µL cell suspension in a sterile 1.5-mL microfuge and add 50 µL trypan blue. Mix gently by repeated pipetting.

g. Add 10 µL of the cell-trypan blue mixture at both ends of the cell counter slide.

h. Determine the viability and cell concentration using Bio-Rad cell counter.

i. To subculture, determine the volume of the cell suspension needed to have $1 \times 10^6$ cells and transfer the volume in T-75 flask. Make the volume to 15-mL with warmed media.

j. Incubate at 37 °C, 5% CO$_2$/95% air.

k. Cells will be ready to subculture for 2-3 days.

6. **Apoptosis analysis**

**Material:**

Annexin V-FITC Apoptosis Detection Kit

**Reference:**

Sigma Technical Bulletin No. MB-390

**Procedure:**
a. Seed 2 x 10^5 cells in each well of the 6-well plate. Make the total volume to 2-mL with warmed growth media.

b. Allow the cells to grow to 80% confluency prior to treatment.

c. Treat the cells leaving wells of untreated cells.

d. After treatment, remove the growth media from the well and wash the cells twice with PBS.

e. Resuspend the cells in 1X binding buffer at a concentration of approximately 1 x 10^6 cells/mL.

f. Add 500 µL of cell suspension to a plastic tube.

g. Add 5 µL of annexin V-FITC and 10 µL of propidium iodide to each cell suspension.

h. Incubate the tubes at room temperature for 10 min and protect from light.

i. Measure the fluorescence of the cells using LSR II flow cytometer. Collect 2-30,000 events.

7. Separation of cellular nucleus and cytoplasm

**Materials:**

Thermo Scientific NE-PER® nuclear and cytoplasmic extraction reagents

Thermo Scientific Halt™ protease inhibitor cocktail kit

Phosphate buffered saline

Trypsin-EDTA solution

**Reference:**

Thermo Scientific Instruction for Product No. 78835

**Procedure:**
a. Perform all centrifugation steps at 4 °C, keep all cell extracts on ice.
b. Add appropriate amount of protease inhibitor in CER I and NER I.
c. For adherent cells, harvest with trypsin-EDTA and centrifuge at 500 x g for 5 min.
d. Wash cells by suspending the cell pellet with PBS.
e. Transfer 1-10 x 10^6 cells to 1.5 mL microcentrifuge tube and pellet by centrifugation at 500 x g for 2-3 min.
f. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible.
g. Add ice-cold CER I to the cell pellet (200 µL for every 20 µL packed cell volume).
h. Vortex the tube vigorously on the highest setting for 15 sec to fully suspend the cell pellet. Incubate tube on ice for 10 min.
i. Add ice-cold CER II (11 µL for every 20 µL packed cell volume) to the tube.
j. Vortex the tube for 5 sec on the highest setting. Incubate tube on ice for 1 min.
k. Vortex the tube for 5 sec on the highest setting and centrifuge it for 5 min at 16,000 x g.
l. Immediately transfer the supernatant (cytoplasmic extract) to a clean pre-chilled tube. Place this tube on ice until use or store at -80 °C for future use.
m. Suspend the insoluble pellet fraction produced in step l in ice-cold NER (100 µL for every 20 µL packed cell volume).
n. Vortex on the highest setting for 15 sec. Place the sample on ice and continue vortexing for 15 sec every 10 min, for a total of 40 min.
o. Centrifuge the tube at 16,000 x g for 10 min.

p. Immediately transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube.

q. Place on ice or store at -80 °C for future use.

8. mRNA extraction

Materials:

Qiagen RNeasy mini kit

96-100% ethanol (do not use denatured alcohol)

Trypsin-EDTA solution

Reference:

Qiagen RNeasy Mini Handbook April 2006

Procedure:

a. Clean the working area with 70% ethanol.

b. Use only sterile, RNase-free pipet tips.

c. Trypsinize the cells, wash twice with PBS to remove any trace of trypsin.

      Pellet the cells by centrifugation.

d. Loosen the cell pellet thoroughly by flicking the tube. Add appropriate amount of RLT Buffer. Vortex or pipet to mix.

e. Homogenize the cell lysate.

f. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting. Do not centrifuge.
g. Transfer up to 700 μL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge for 15 sec at >/ 8,000 x g. Discard the flow-through.

h. Add 700 μL Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 sec at >/ 8,000 x g to wash the spin column membrane. Discard the flow-through. (Skip this step if on-column DNase digestion will be performed)

i. Add 500 μL Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15 sec at >/ 8,000 x g to wash the spin column membrane. Discard the flow-through.

j. Add 500 μL Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 2 min at >/ 8,000 x g to wash the spin column membrane.

k. Optional: Place the RNeasy spin column in a new 2 mL collection tube and discard the old collection tube with the flow-through. Close the lid gently and centrifuge at full speed for 1 min.

l. Place the RNeasy spin column in a new 1.5 mL collection tube. Add 30-50 μL RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at >/ 8,000 x g to elute the RNA.

m. Step l may be repeated if desired (i.e. if expected RNA yield is > 30 μg.

9. **On-Column DNase digestion**

   **Materials:**
   
   Qiagen RNase-Free DNase set

   **Reference:**
Qiagen RNeasy Mini Handbook April 2006

Procedure:

a. Prepare DNase I stock solution before using the RNase-Free DNase set for the first time. Dissolve the lyophilized DNase I in 550 µL of the RNase-free water. Inject the RNase-free water into the vial to avoid loss of DNase I. Mix gently by inverting the vial. Do not vortex.

b. Aliquot the DNase I stock solution and store at -20 °C for 9 months.

c. Add 350 µL Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 sec at >/ 8,000 x g to wash the spin column membrane.

Discard the flow-through.

d. Add 10 µL DNase I stock solution to 70 µL Buffer RDD. Mix by gently inverting the tube and centrifuge briefly to collect residual liquid from the sides of the tube.

e. Add the DNase I incubation mix (80 µL) directly to the RNeasy spin column membrane and place on the benchtop (20-30 °C) for 15 min.

f. Add 350 µL Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 sec at >/ 8,000 x g to wash the spin column membrane.

Discard the flow-through. Continue with the first Buffer RPE wash step in the relevant protocol.

10. Immunofluorescence microscopy for lunasin internalization study

Materials:

8-well IBIDI plate

Phenol-red DMEM
4% paraformaldehyde in PBS
Lunasin mouse monoclonal antibody
ITsignal FX blocking solution
Goat anti-mouse conjugate Alexa Fluor 488
DAPI
PBS
Prolong gold
HPLC-grade methanol

**Procedure:**

a. Plate 1000-2000 cells per well in 8-well IBIDI plate in 10% phenol red-free DMEM.

b. Incubate until the cells reached 50-60% confluence.

c. Treat cells with 1 µM lunasin or PBS as blank for 24 h.

d. Aspirate the media and wash cells with fresh phenol red-free medium.

e. Wash cells in PBS (room temperature) three times quickly to remove debris and serum.

f. Fix all wells in 4% paraformaldehyde in PBS for 30 min at room temperature.

g. Wash wells with PBS 3x for 5 min each at room temperature.

h. Add 0.5% Triton-X 100 in PBS for 15 min at room temperature.

i. Wash once with PBS at room temperature.

j. Add ultracold HPLC-grade methanol and incubate the whole dish at -20 °C for 15 min.
k. Remove the methanol one by one and replace with PBS. Incubate in PBS for 30 min at room temperature.
l. Block each well with 4-5 drops ITsignal FX for 30 min at room temperature.
m. Rinse wells once with PBS. Leave wells that will not receive primary antibody in ITsignal FX.
n. Prepare 1:100 dilution of lunasin mouse monoclonal antibody in PBS, add one drop of ITsignal FX and centrifuge for 5 min at 12,000 rpm. Add the supernatant to wells receiving primary antibody. Incubate at 37 °C for 2 h.
o. Wash wells with PBS 3x for 5 min each. All succeeding steps should be done in the dark and place the 8-well plate in a small box.
p. Dilute goat anti-mouse Alexa fluor 488 secondary antibody in PBS, add two drops of ITsignal FX for every 2-mL and centrifuge for 5 min at 12,000 rpm. Add supernatant to wells receiving secondary antibody. Leave wells not receiving secondary antibody in ITsignal FX.
q. Wash all wells with PBS 3x for 5 min each.
r. Incubate all wells in DAPI (4 µL stock + 1,996 µL PBS for every 8-well plate) for 15 min.
s. Wash all plates briefly in PBS 3x.
t. Prior to use, make sure that Prolong gold is at room temperature. Dry each well with a Kimwipes (pointed part) making sure that all well is dry as possible without trapping a bubbles. Mount all wells with prolong gold approximately 120 µL per well.
u. Keep the dish in the dark with lid loosely on top at room temperature for 24 h.

v. Store in dark at 4 °C with lids closed or sealed with parafilm until analysis.

w. Set up an appointment for confocal microscopy at The Institute for Genomic Biology.

11. Immunohistochemical staining of paraffin-embedded tissue

**Materials:**

ABC staining kit from Santa Cruz Biotechnology

Appropriate primary antibody

PBS

Hydrogen peroxide

Hematoxylin

Xylene

Ethanol

**Reference:**

ImmunoCruz ABC staining system product datasheet

**Procedure:**

a. Dry paraffin-embedded sections by placing in a 60 °C oven for 30-60 min.

b. Prepare slides by washing with xylenes (3x for 3 min), 100% alcohol (3x for 3 min), 95% alcohol (1x for 3 min) and deionized water (1x for 3-5 min)

c. Retrieve antigen by microwaving for 3 min.

d. Incubate slides in 0.1-1% H₂O₂ diluted in PBS for 5-10 min.

e. Wash with PBS 2x for 5 min each.
f. Incubate sections in 1.5% blocking serum in PBS for 1 h.

g. Remove the blocking solution and incubate with primary antibody (1:50 dilution) diluted in 1.5% blocking solution at 4 °C overnight or at room temperature for 2-3 h. For sections not receiving the primary antibody, leave on 1.5% blocking solution.

h. Wash with PBS for 5 min each.

i. Incubate sections for 30 min with biotinylated secondary antibody (1 µg/mL).

j. Wash with 3 changes of PBS for 5 min each.

k. Incubate sections with AB enzyme reagent for 30 min.

l. Wash with 3 changes of PBS for 5 min each.

m. Incubate sections in 1-3 drops peroxidase substrate for 10 min.

n. Wash sections in deionized water for 5 min.

o. Counterstain with hematoxylin for 10 sec.

p. Wash in running deionized water for 5 min.

q. Dehydrate sections as follows: 95% ethanol (2x for 10 sec each), 100% ethanol (2x for 10 sec each) xylene (3x for 10 sec each).

r. Mount in permanent mounting medium.

s. Allow to dry overnight.

t. Scan using Nanozoomer Digital Pathology at The Institute for Genomic Biology.

u. Quantify staining intensity using Teton machine Axio Vision program at The Institute for Genomic Biology.

12. Hematoxylin and Eosin staining of paraffin-embedded tissue
**Materials:**

Hematoxylin

Eosin

Xylene

Ethanol

Clarifier

**Procedure:**

a. Dry slides in 60 °C oven for 30-60 min.

b. Dip all slides in 3 changes of xylenes for 3 min each.

c. Dip all slides in 3 changes of 100% ethanol for 3 min each.

d. Dip all slides in 95% ethanol for 3 min.

e. Rinse with deionized water for 3 min.

f. Dip all slides in hematoxylin for 1 min (max 4 min).

g. Dip in clarifier solution (should be fresh each time) for 1 min.

h. Wash in running tap water for 3-5 min.

i. Dip all slides in eosin for 1 min (same time as in hematoxylin).

j. Dip in three changes of 100% ethanol for 2 min each.

k. Dip in 3 changes of xylene for 2 min each.

l. Coverslip with Permount mounting media.

m. Dry all slides overnight and scan using Nanozoomer Digital Pathology at The Institute for Genomic Biology.
CURRICULUM VITAE

EDUCATION

Ph D  Food Science and Human Nutrition
2007-2011 Department of Food Science and Human Nutrition
University of Illinois at Urbana-Champaign, Urbana IL
Dissertation Title: Soybean lunasin mediates colon carcinogenesis
by inducing apoptosis and preventing outgrowth of metastasis

MS  Food Science (minor: Biochemistry)
2002-2005 Institute of Food Science and Technology
University of the Philippines Los Banos, Los Banos Laguna

BS  Food Technology
1997-2001 Institute of Food Science and Technology
University of the Philippines Los Banos, Los Banos Laguna

WORK EXPERIENCE

Graduate Research Assistant
University of Illinois at Urbana-Champaign 2007-2011

Graduate Teaching Assistant
University of Illinois at Urbana-Champaign 2008-2011

Faculty
University of the Philippines Los Banos 2001-2007

Quality Assurance Analyst

Summer Intern
Nugget Food Corporation April – May 2000

RECOGNITIONS, HONORS AND AWARDS

Hans Kaunitz Award, USA Section - American Oil Chemists’ Society, May 2011

Outstanding Teaching Assistant, Incomplete List of Teachers Rank as Excellent by
their students, Fall 2010, University of Illinois at Urbana-Champaign, Daily Illini

Graduate Student Research Award (Ph D), College of Agricultural, Consumer and
Environmental Sciences, University of Illinois at Urbana-Champaign, 2011
Outstanding Teaching Assistant, Incomplete List of Teachers Rank as Excellent by their Students, Spring 2010, University of Illinois at Urbana-Champaign, Daily Illini

Henry D. and Donna E. Strunk Fellowship Merit Award, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, May 2010

Outstanding Teaching Assistant, Incomplete List of Teachers Rank as Excellent by their Students, Fall 2009, University of Illinois at Urbana-Champaign, Daily Illini

Outstanding Ph D student, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, May 2009

Outstanding Teaching Assistant, Incomplete List of Teachers Rank as Excellent by their Students, Fall 2008, University of Illinois at Urbana-Champaign, Daily Illini

Henry D. and Donna E. Strunk Fellowship Merit Award, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, May 2009

Henry D. and Donna E. Strunk Fellowship Merit Award, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, May 2008

Toshido Nishida Research Award, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, April 2008

International Publication Awards, University of the Philippines, Diliman Quezon City March 2006

Development Fund Faculty Grant, University of the Philippines Los Baños, July 30, 2004 – June 30, 2005 (Preparation of Lecture Syllabus in Food Chemistry)

Magna Cum Laude, University of the Philippines Los Banos, May 2001

Department of Science and Technology – Science Education Institute Scholarship, June 1997 – April 2001

Gamma Sigma Delta Honor Society of Agriculture, Outstanding Junior Student, March 2000

PUBLICATIONS


de Mejia EG, Dia VP. 2009. Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-kB pathway in the macrophage. Peptides. 30: 2388-2398


POSTER AND ORAL PRESENTATIONS

Dia, VP., Gonzalez-de Mejia, E. Lunasin causes cytotoxicity in human colon cancer cells through activation of apoptosis and induction of nuclear clusterin expression. 101st American Oil Chemical Society Meeting, May 15-18, 2010. Phoenix, Arizona, USA.

Dia, VP. Vegetables and Pancreatic Cancer, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Journal Club Diet and Cancer Seminar Series, Spring Semester 2010

Gonzalez de Mejia, E., Dia, VP. Anti-inflammatory activity of naturally present soybean peptides, 100th American Oil Chemical Society Meeting, May 3-6, 2009 Orlando Florida, USA.


Dia, VP. Cyclooxygenase-2 and Colon Cancer: The Big Connection, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Journal Club Diet and Cancer Seminar Series, Fall Semester 2007

Dia, VP. Isoflavones and Colorectal Cancer, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Journal Club Diet and Cancer Seminar Series, Spring Semester 2008


University of the Philippines Los Baños

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Student Member, American Oil Chemical Society  
Student Member, Institute of Food Technologists  
Member, Philippine Association of Food Technologists Region IV Chapter  
Member, Gamma Sigma Delta Honor Society of Agriculture  
Member, Kapisanang Kimika ng Pilipinas Timog Katagalugan (Chemical Society of the Philippines Southern Tagalog Chapter)
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